US008911998B2

(12) **United States Patent**
Croce et al.

(10) **Patent No.: US 8,911,998 B2**
(45) **Date of Patent: Dec. 16, 2014**

(54) **METHODS FOR IDENTIFYING FRAGILE HISTIDINE TRIAD (FHIT) INTERACTION AND USES THEREOF**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1216 days.

(21) Appl. No.: **12/739,541**

(22) PCT Filed: **Oct. 27, 2008**

(86) PCT No.: **PCT/US2008/081294**

§ 371 (c)(1),
(2), (4) Date: **Feb. 25, 2011**

(87) PCT Pub. No.: **WO2009/055773**

PCT Pub. Date: **Apr. 30, 2009**

(65) **Prior Publication Data**

US 2012/0010092 A1 Jan. 12, 2012

Related U.S. Application Data

(60) Provisional application No. 61/000,480, filed on Oct. 26, 2007.

(51) **Int. Cl.**

C12N 15/09	(2006.01)
A61K 38/17	(2006.01)
G01N 33/50	(2006.01)
C12N 15/86	(2006.01)
C12Q 1/68	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/86*** (2013.01); *C12N 2710/10343* (2013.01); ***A61K 38/17*** (2013.01); ***G01N 33/5011*** (2013.01); *C12Q 2600/136* (2013.01); *G01N 2333/90293* (2013.01); ***C12Q 1/6886*** (2013.01); *C12Q 2600/106* (2013.01)

USPC **435/455**

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

Provided herein are methods and compositions for the diagnosis, prognosis and treatment of a cancer associated disorder using the Fhit gene.

4 Claims, 37 Drawing Sheets
(4 of 37 Drawing Sheet(s) Filed in Color)

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Wiemer et al., "The Role of MicroRNAs in Cancer: No Small Matter," European Journal of Cancer, Jun. 12, 2007, vol. 43, No. 10, pp. 1529-1544.

Xi, Y. et al., "Prognostic Values of MicroRNAs in Colorectal Cancer," Biomarker Insights, Jan. 2006, pp. 113-121, vol. 1.

Zhao et al., "p53 Mediates the Negative Regulation of MDM2 by Orphan Receptor TR3," The EMBO Journal, 2006, pp. 5703-5715, vol. 25.

* cited by examiner

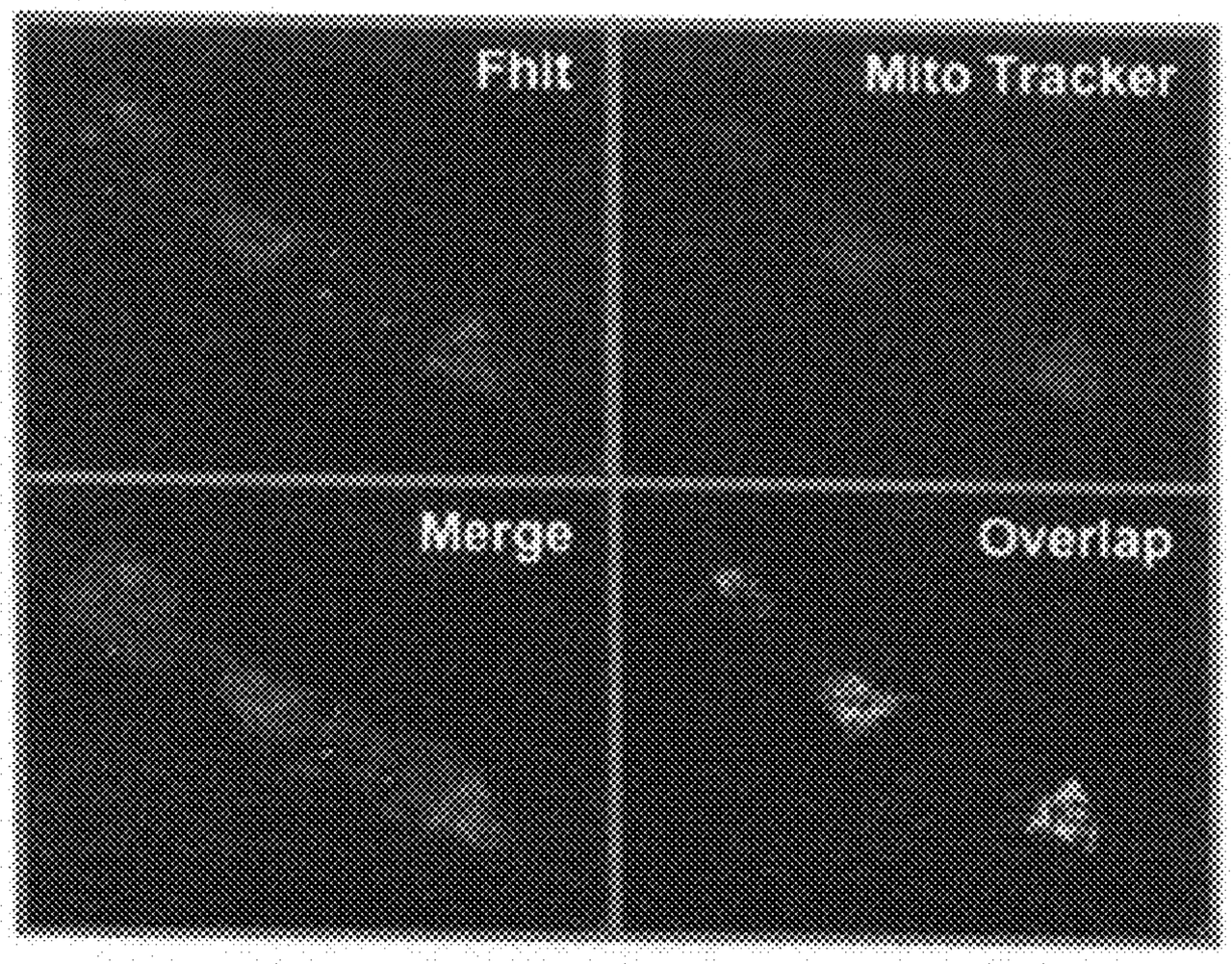


Figure 1A

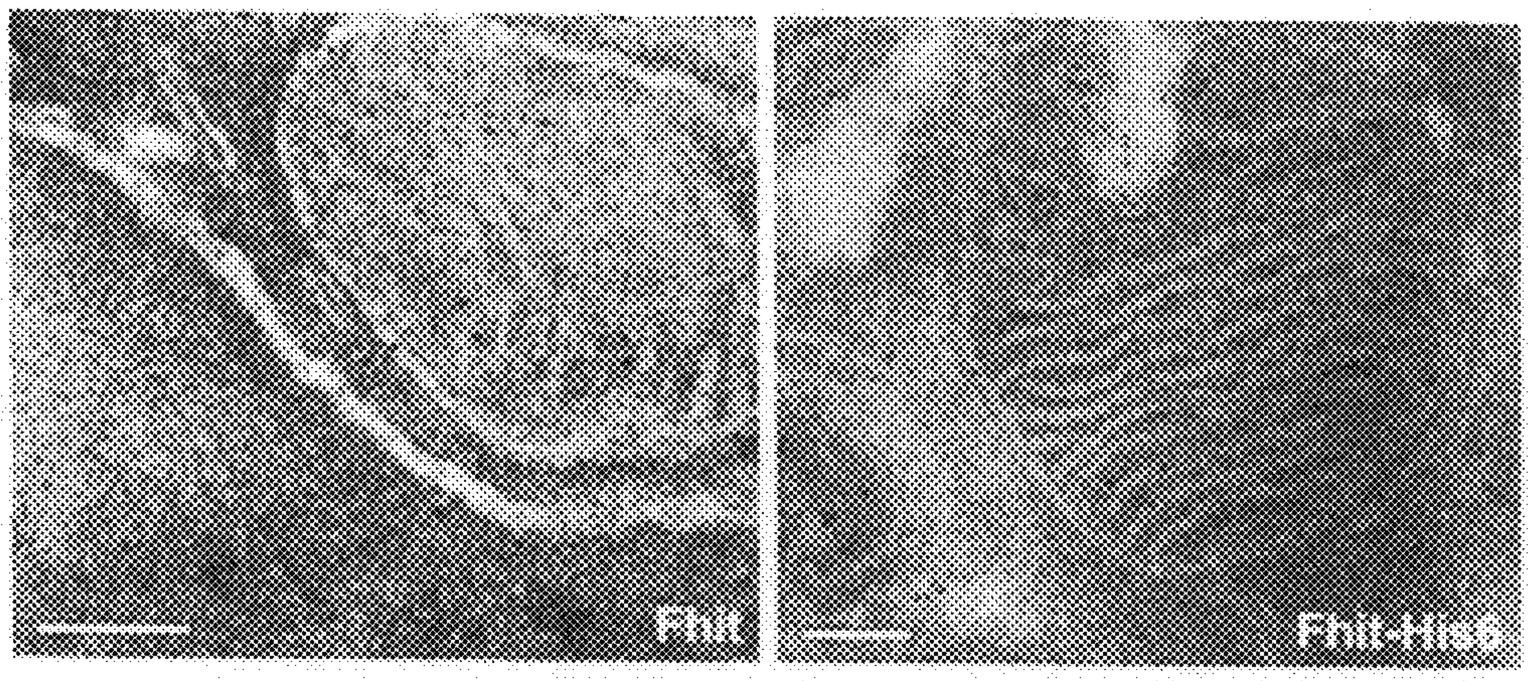


Figure 1B

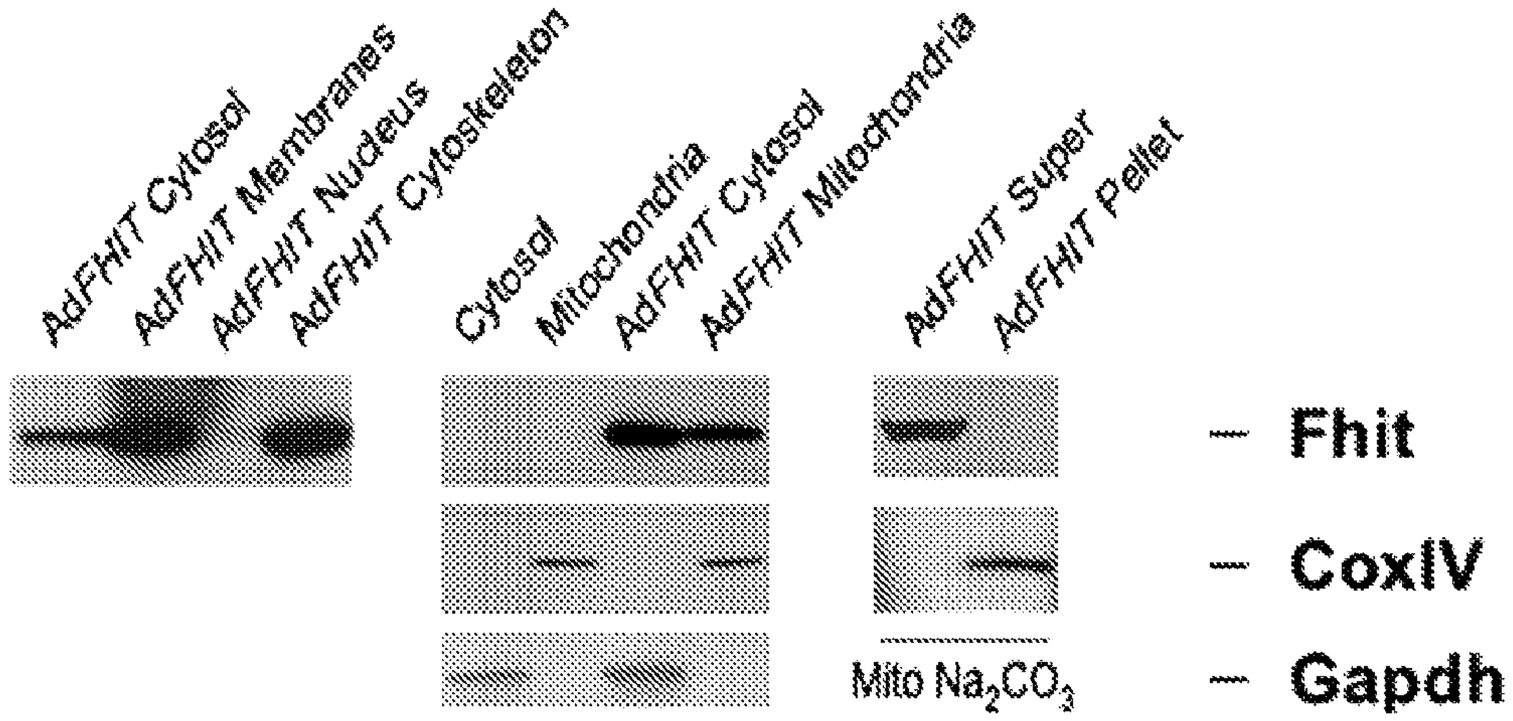


Figure 1C Figure 1D Figure 1E

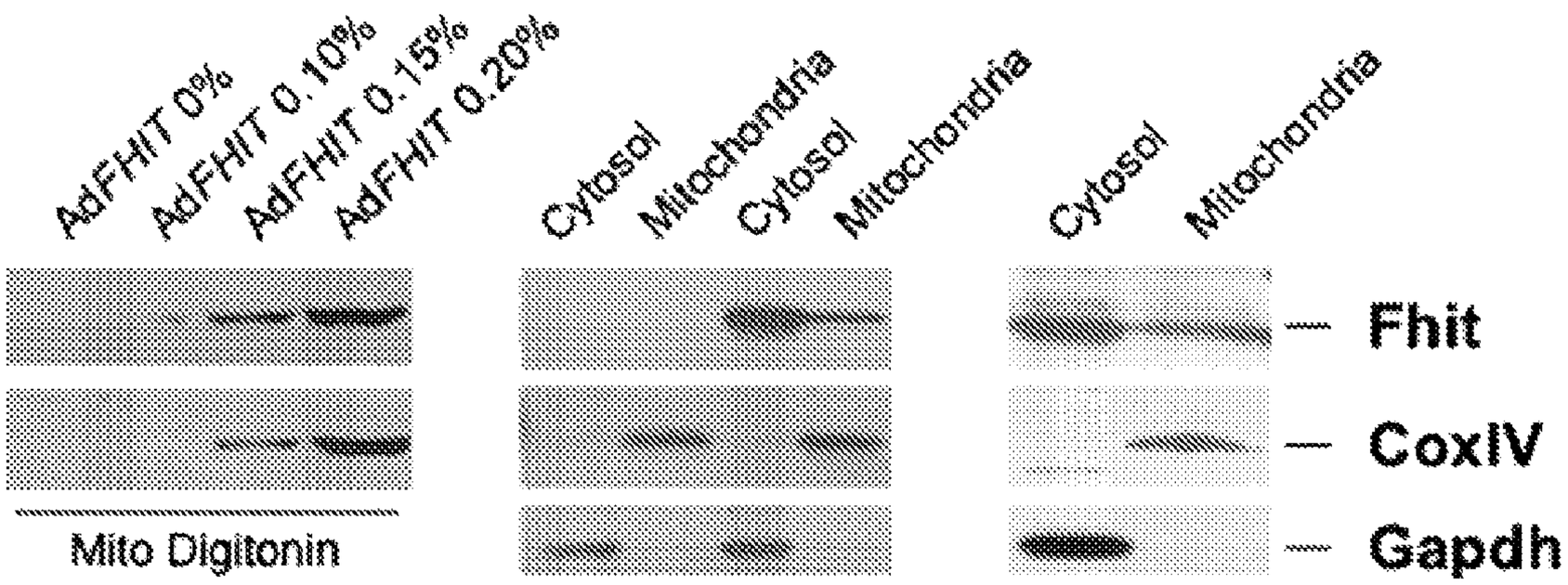


Figure 1F Figure 1G Figure 1H

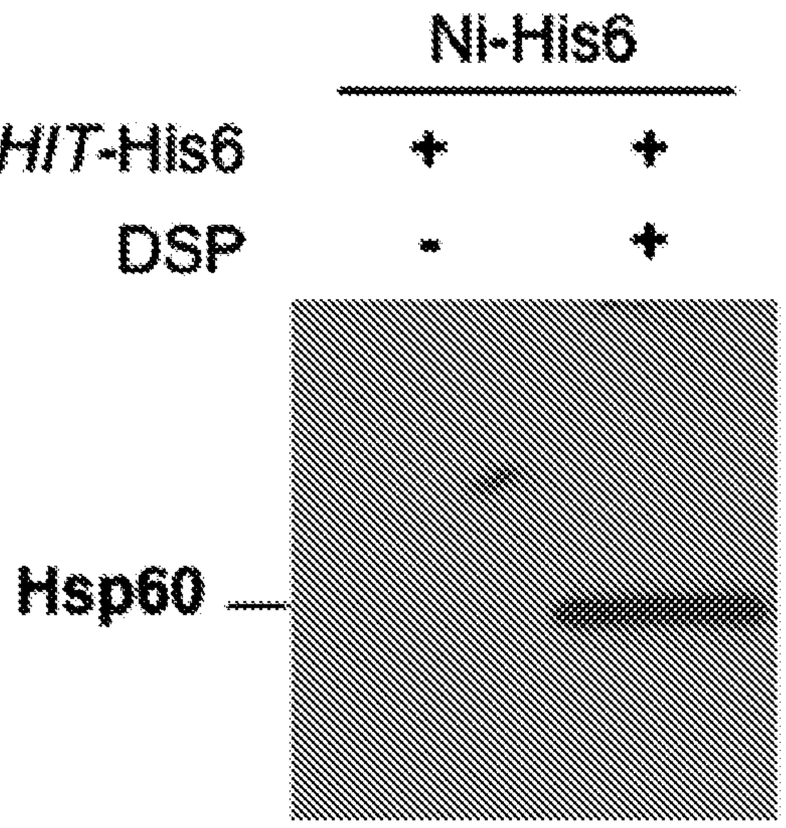


Figure 2A

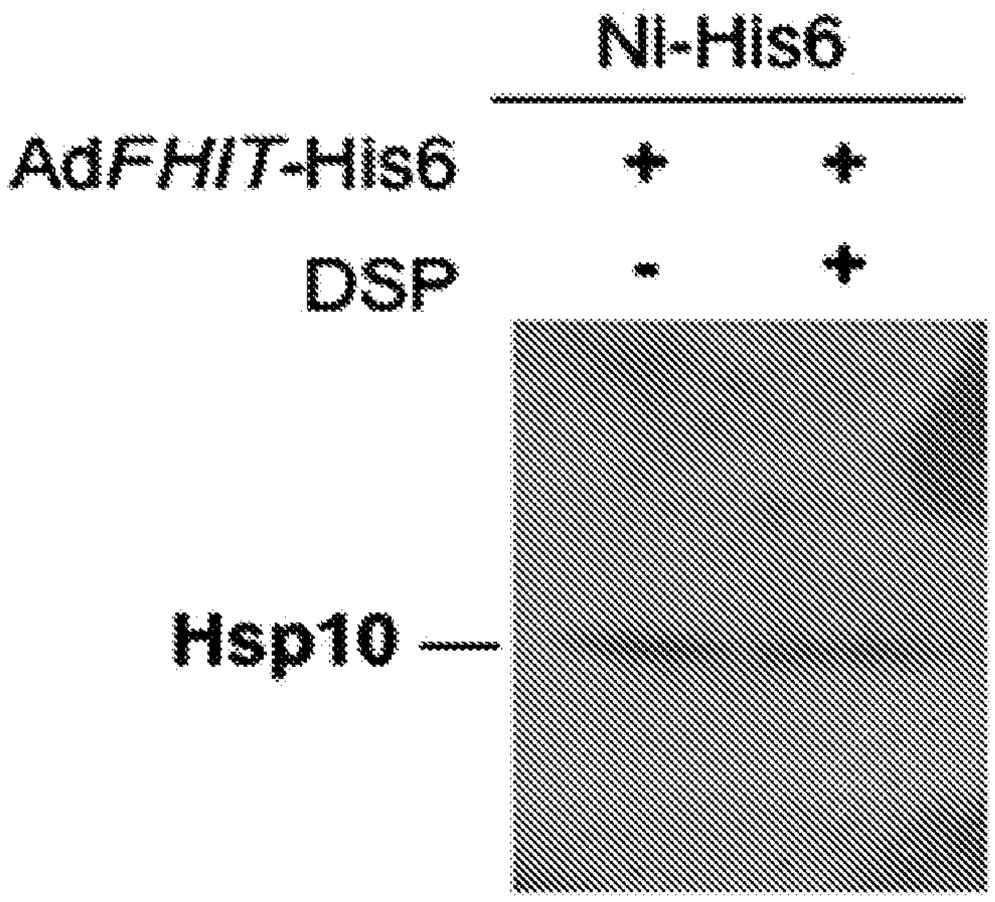


Figure 2B

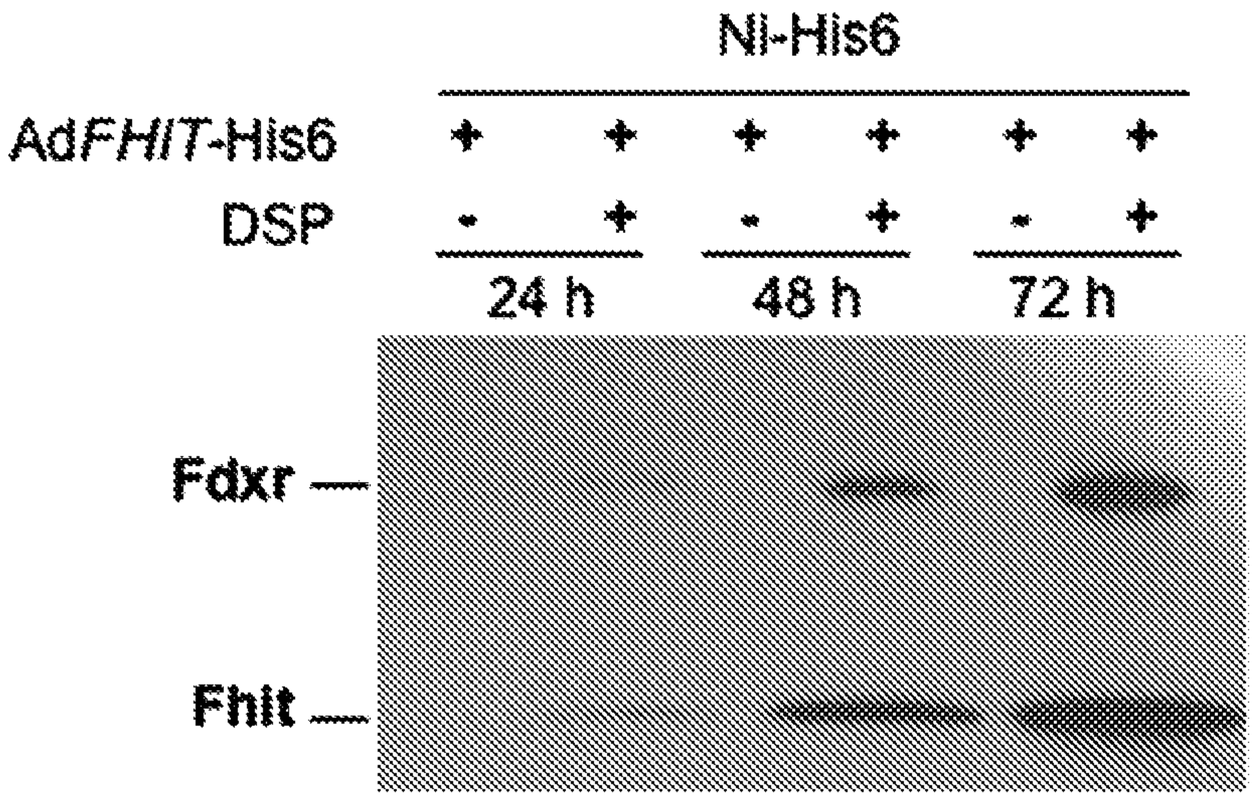


Figure 2C

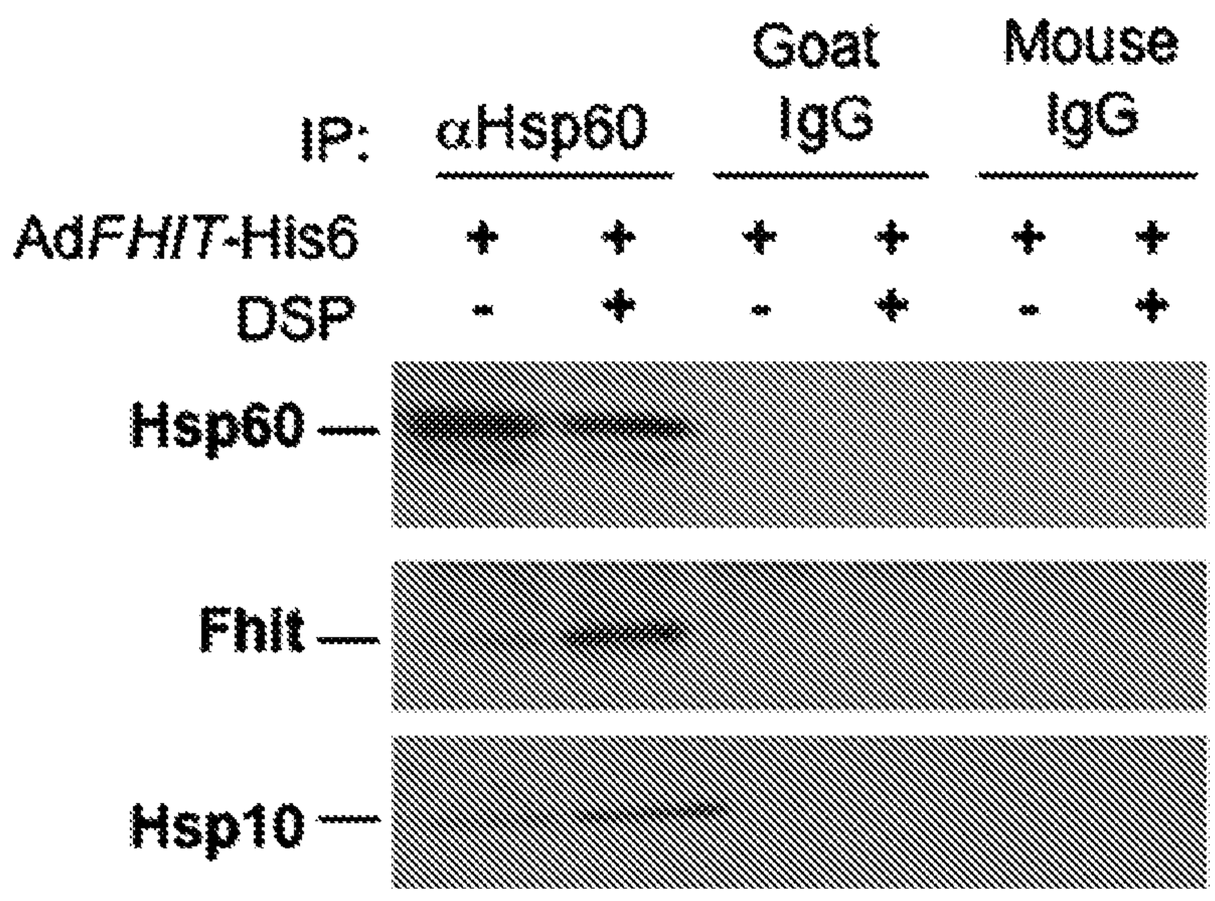


Figure 2D

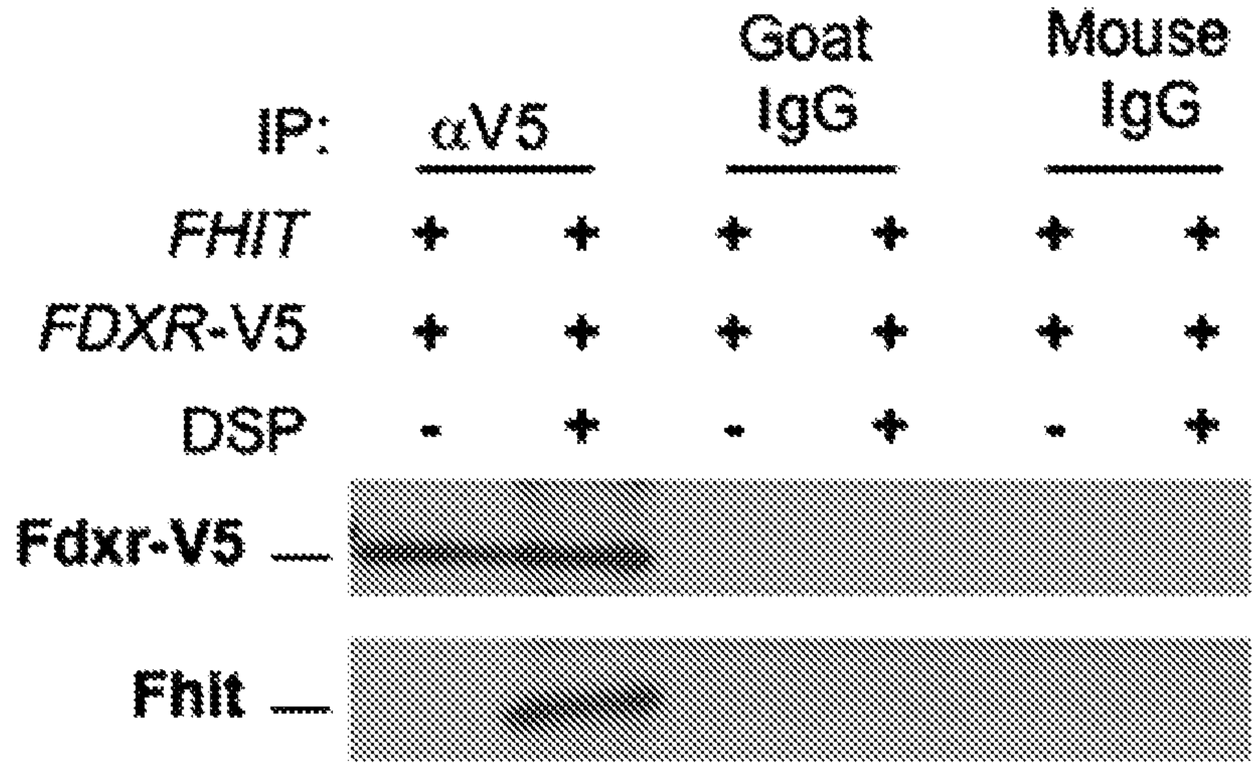


Figure 2E

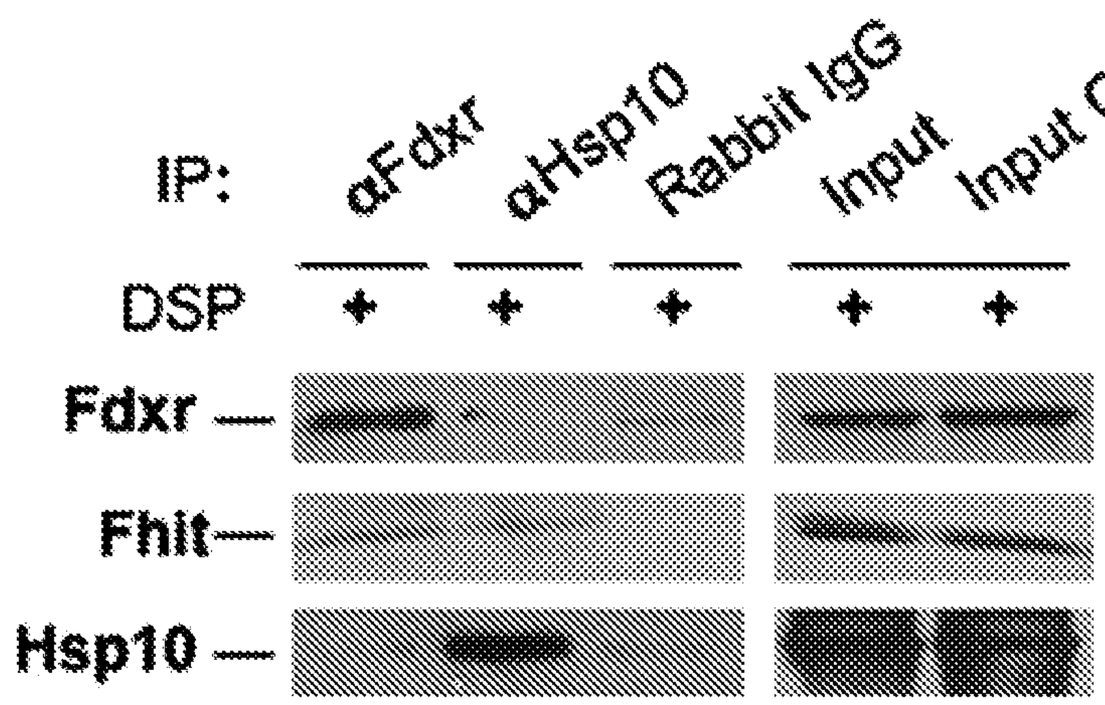


Figure 2F

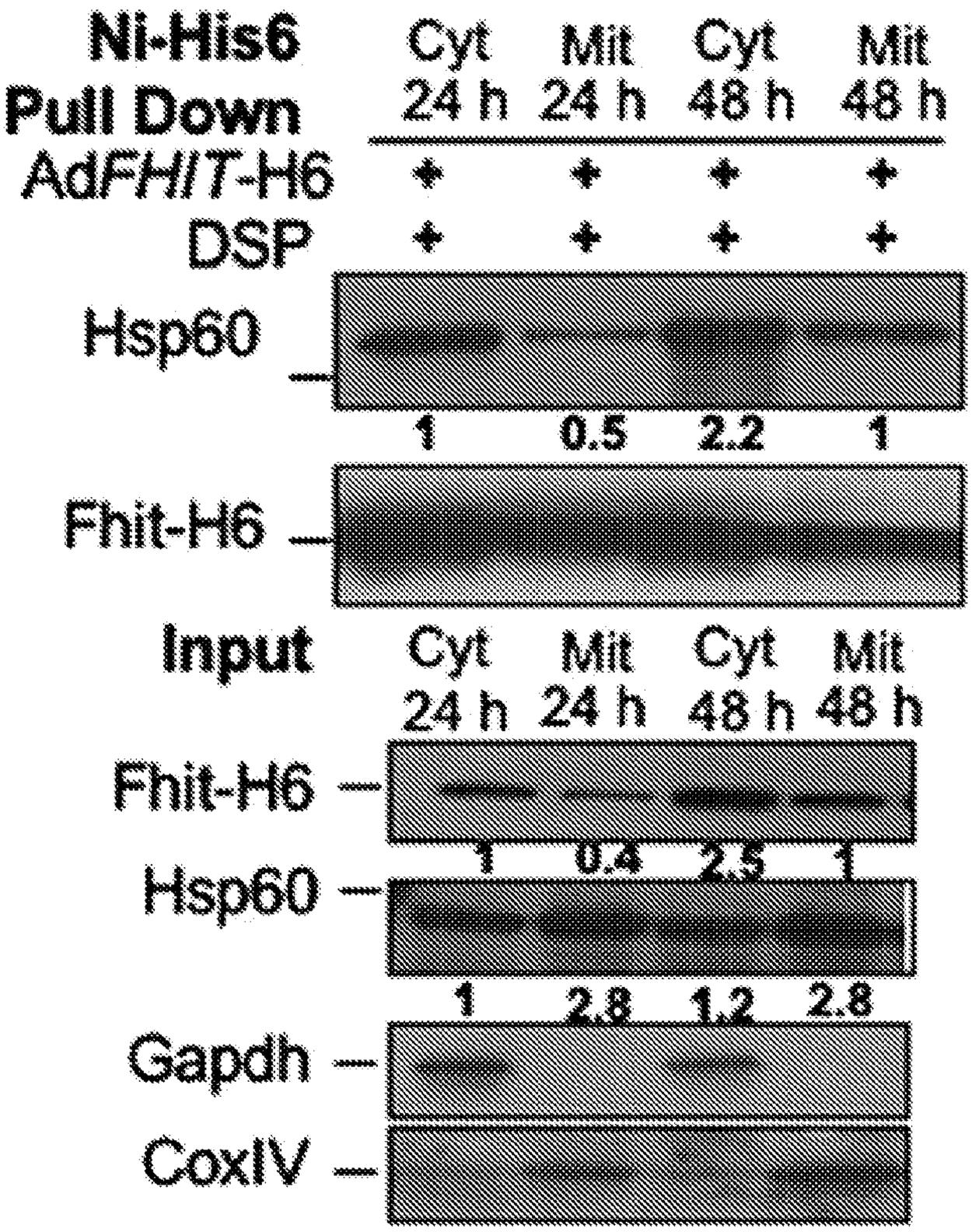

Ni-His6 Pull Down
Cyt 24 h
Mit 24 h
Cyt 48 h
Mit 48 h
AdFHIT-H6
DSP
Hsp60
1
0.5
2.2
1
Fhit-H6
Input
Cyt 24 h
Mit 24 h
Cyt 48 h
Mit 48 h
Fhit-H6
1
0.4
2.5
1
Hsp60
1
2.8
1.2
2.8
Gapdh
CoxIV

Figure 3A

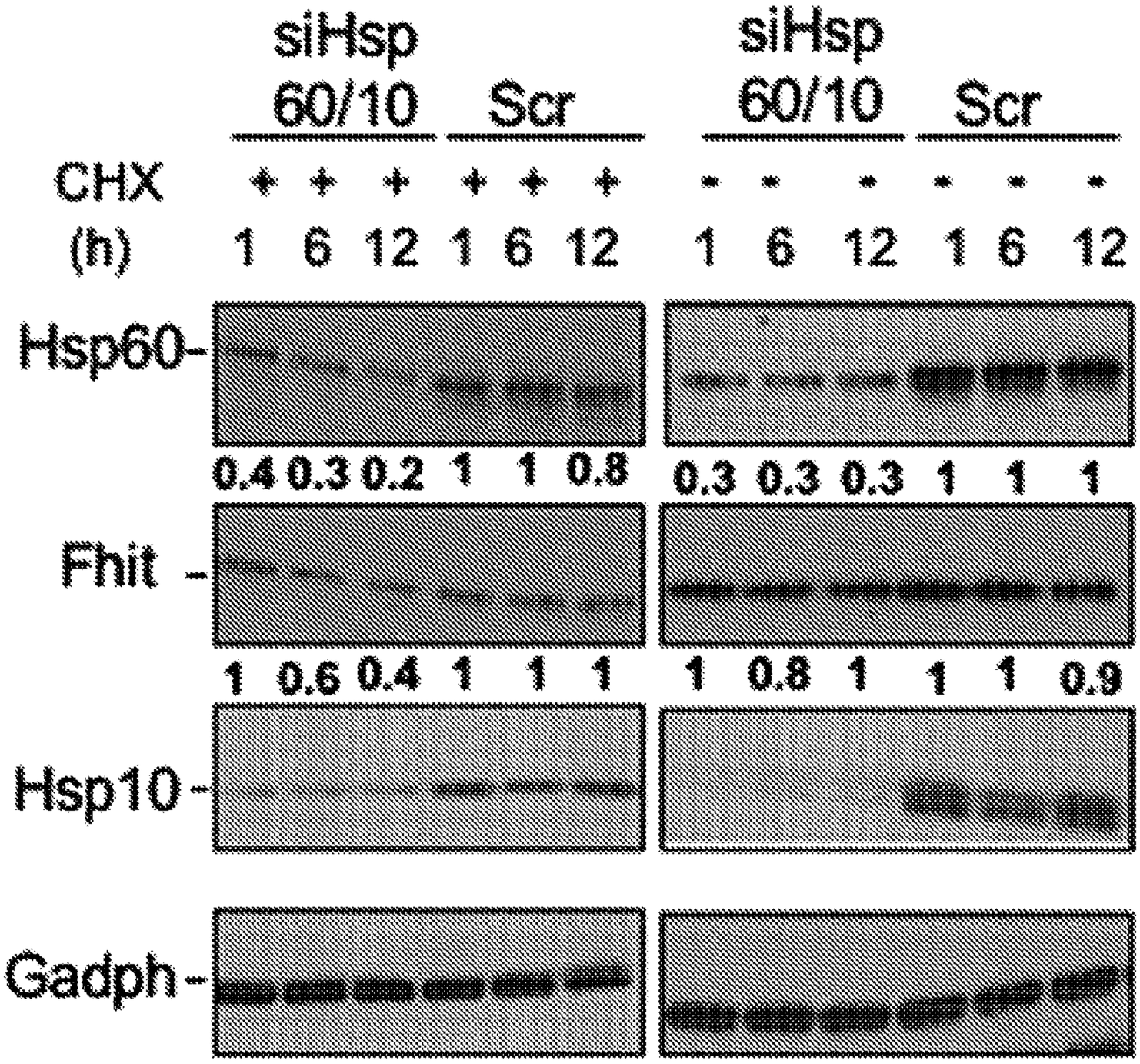

siHsp 60/10
Scr
siHsp 60/10
Scr
CHX + + + + + + - - - - - -
(h) 1 6 12 1 6 12 1 6 12 1 6 12
Hsp60-
0.4 0.3 0.2 1 1 0.8
0.3 0.3 0.3 1 1 1
Fhit -
1 0.6 0.4 1 1 1
1 0.8 1 1 1 0.9
Hsp10-
Gadph-

Figure 3B

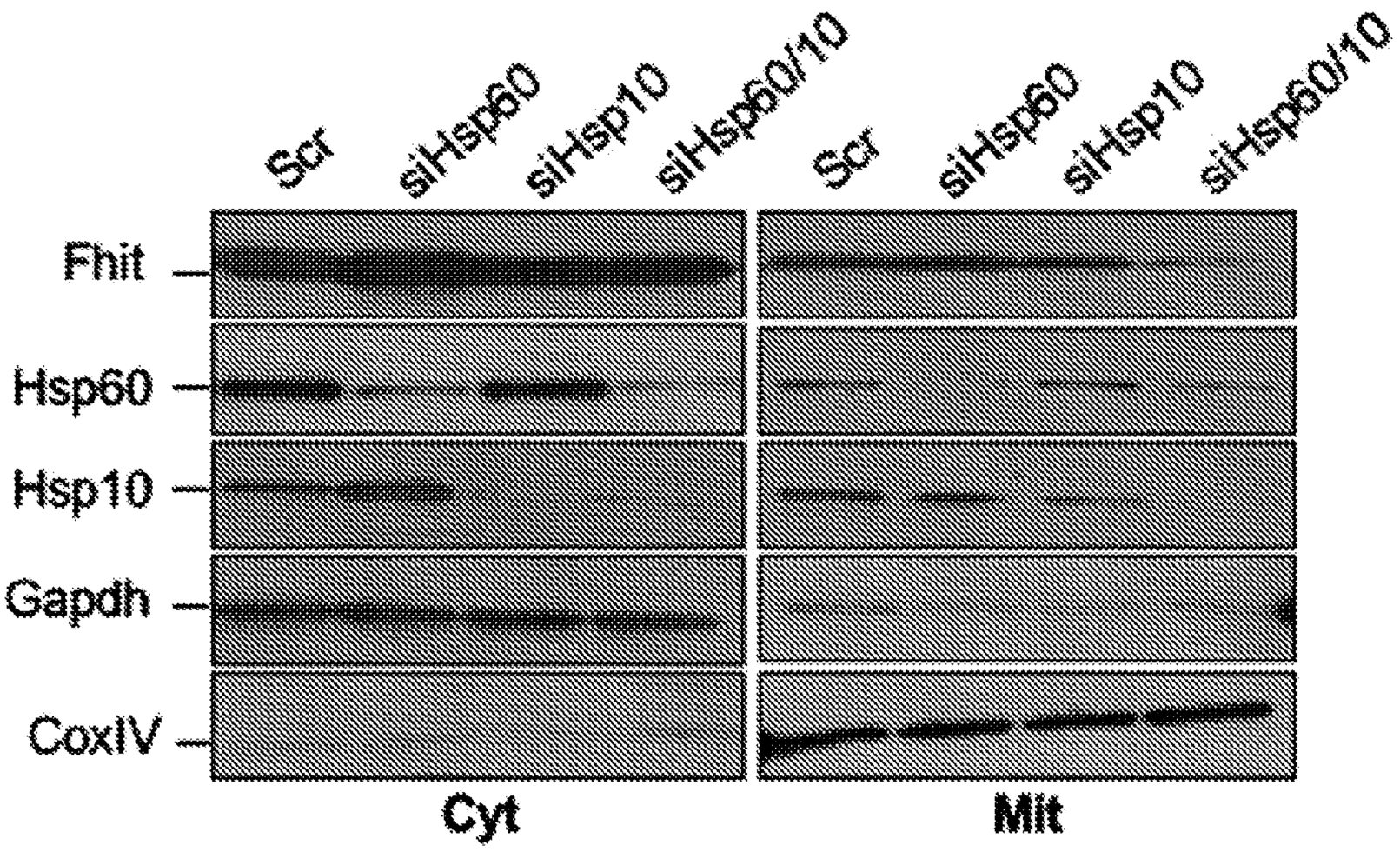


Figure 3C

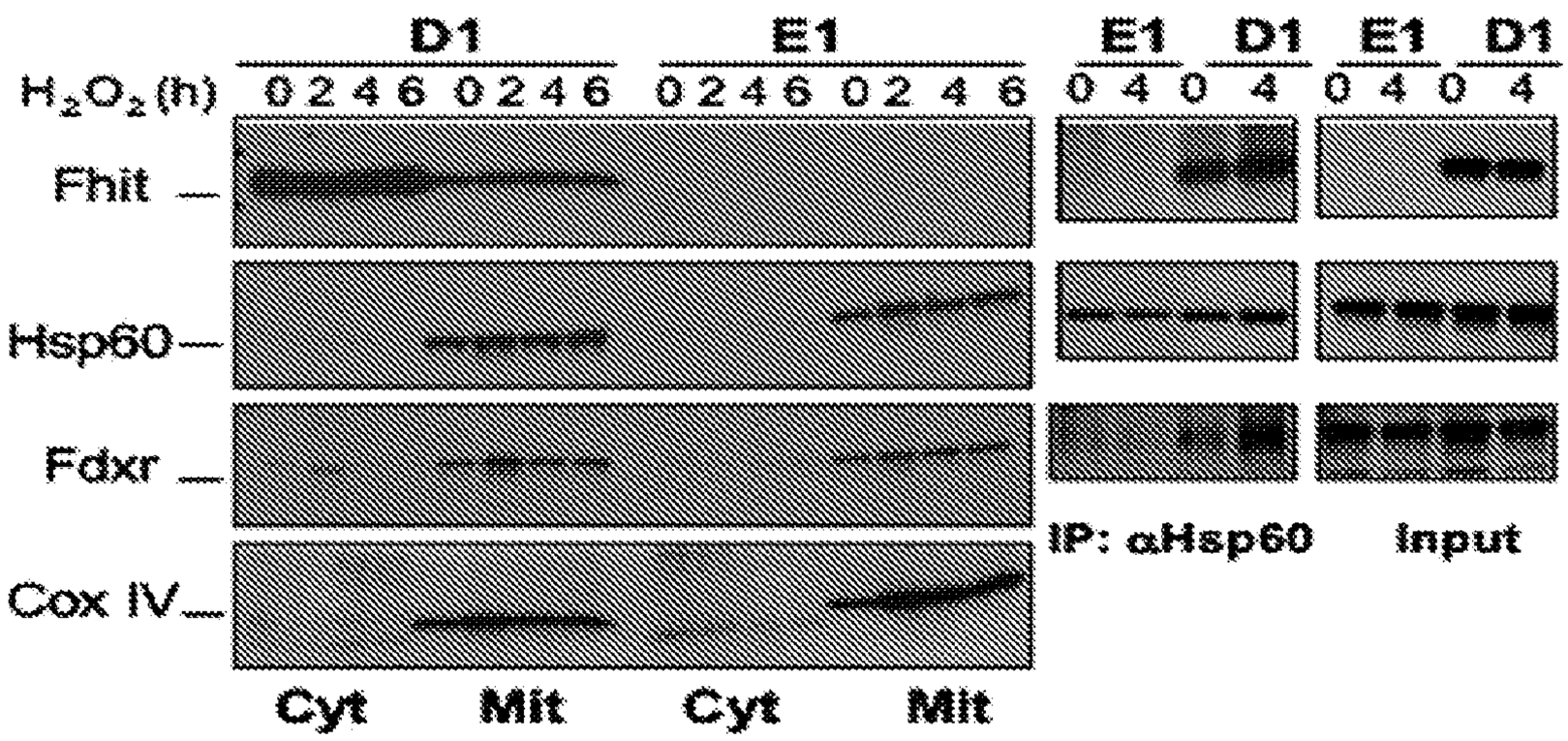


Figure 3D

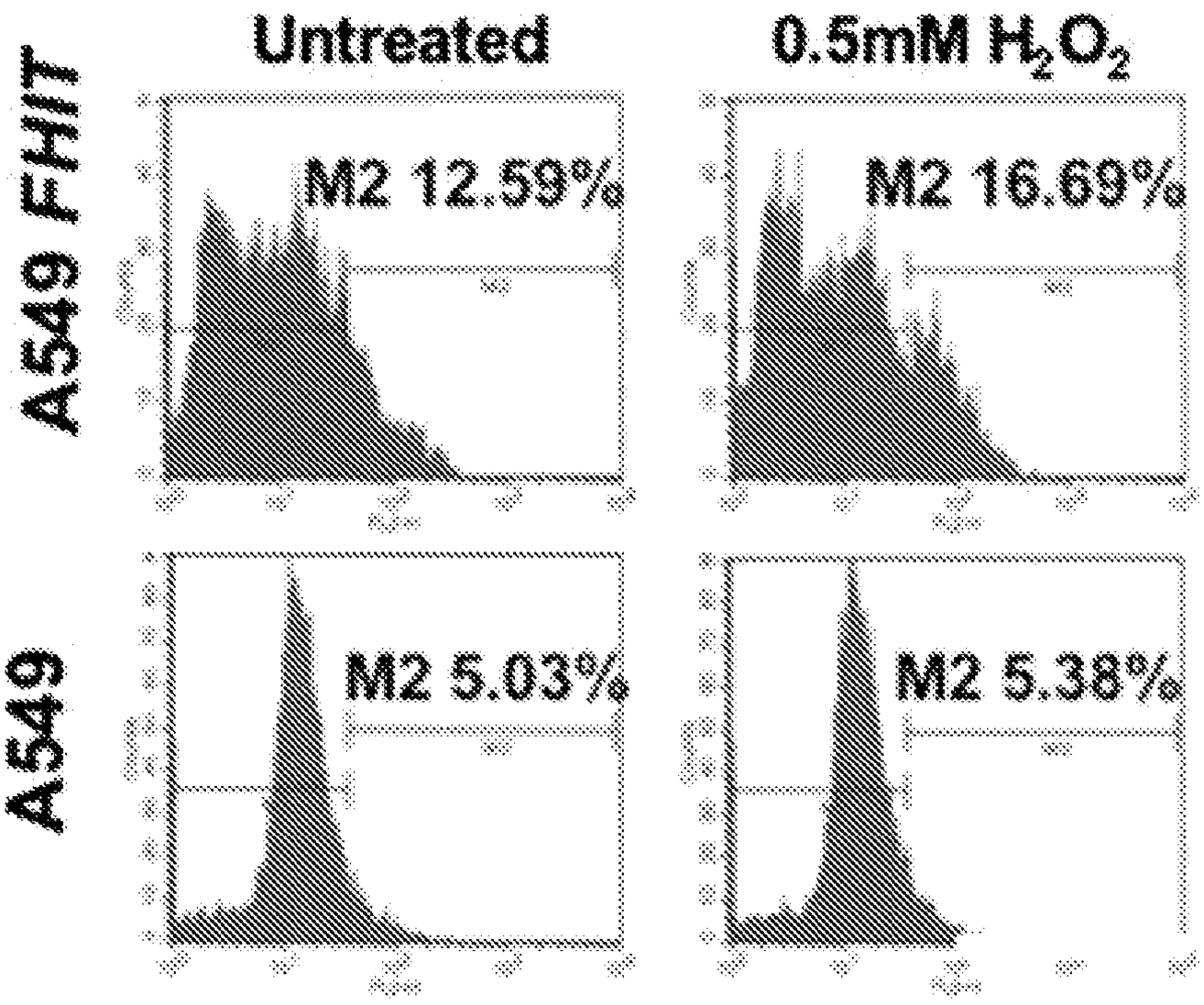


Figure 4A

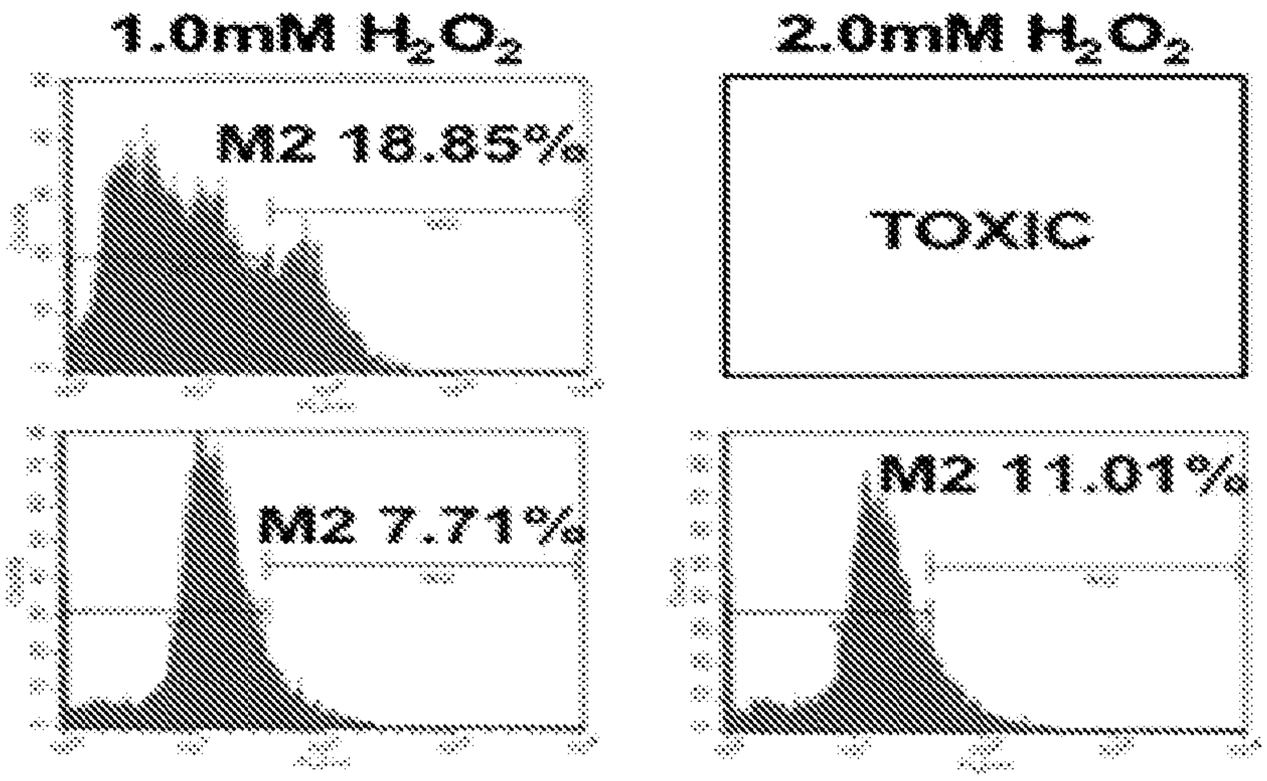


Figure 4A cont.

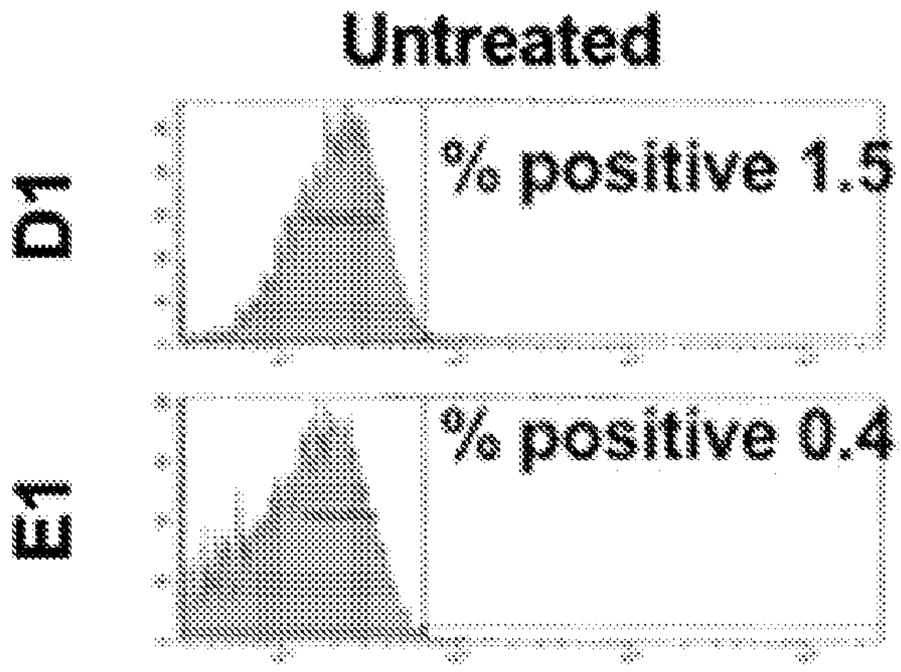


Figure 4B

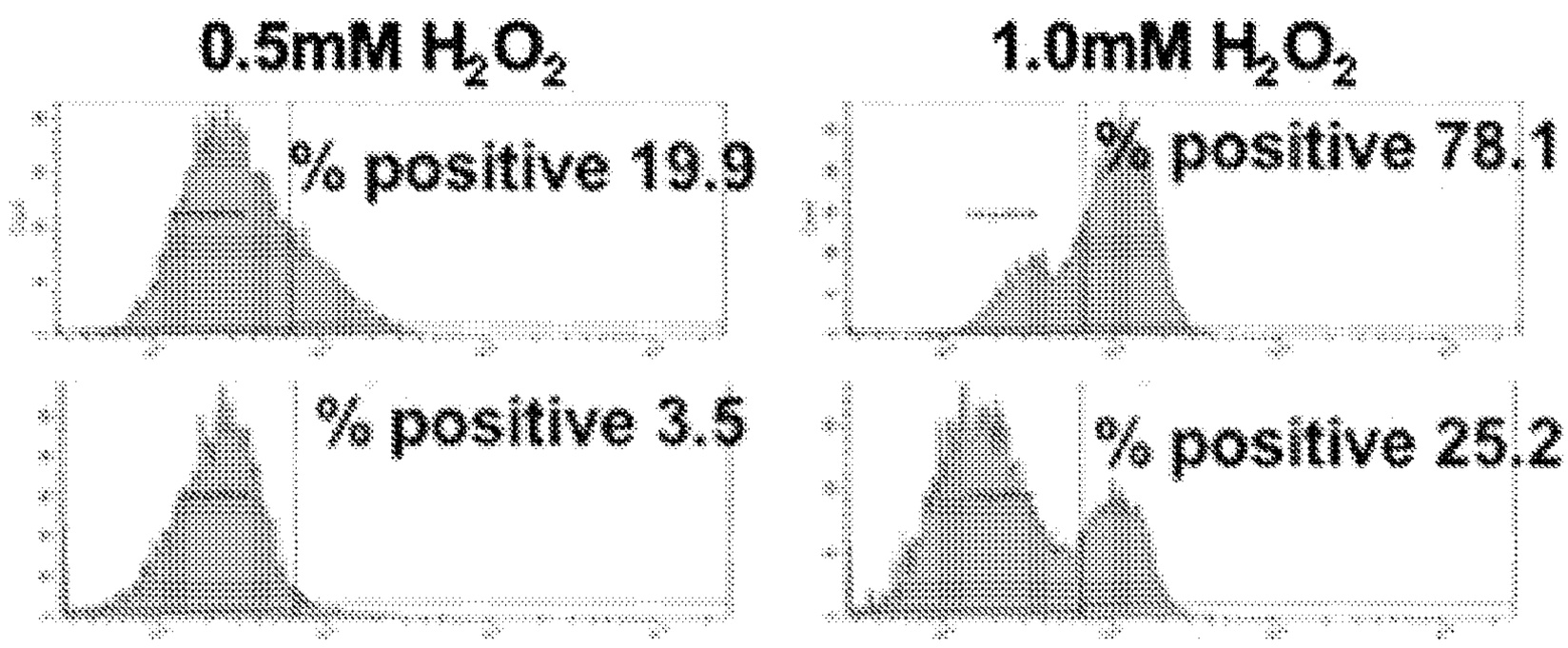


Figure 4B cont.

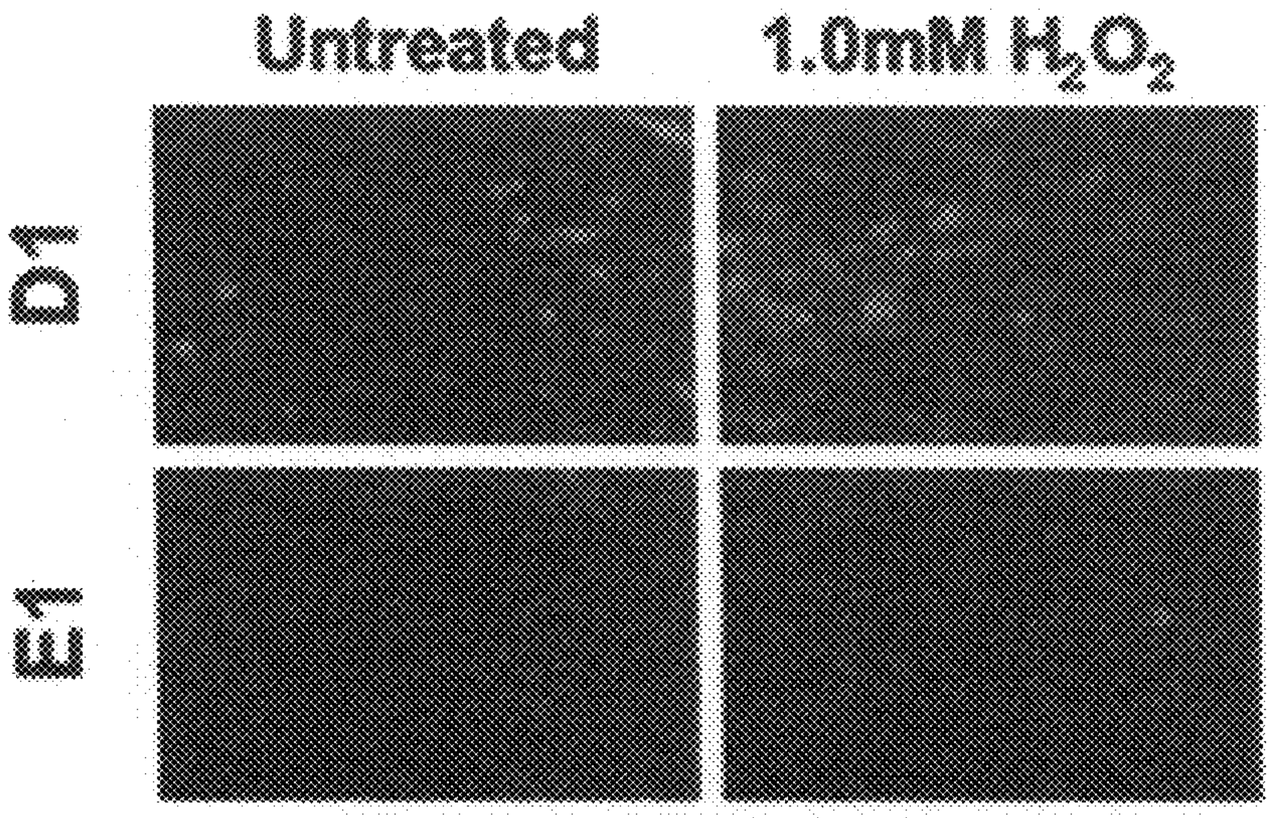


Figure 4C

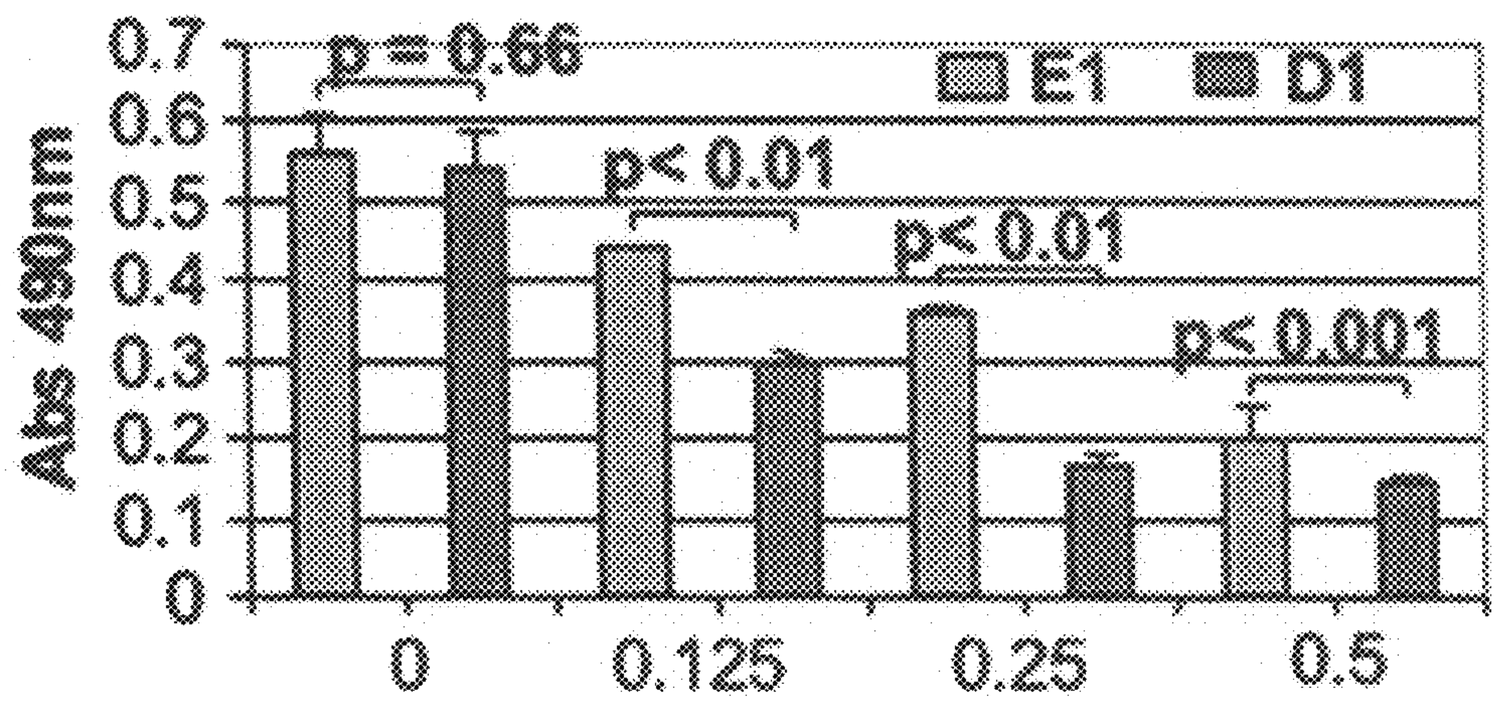


Figure 4D

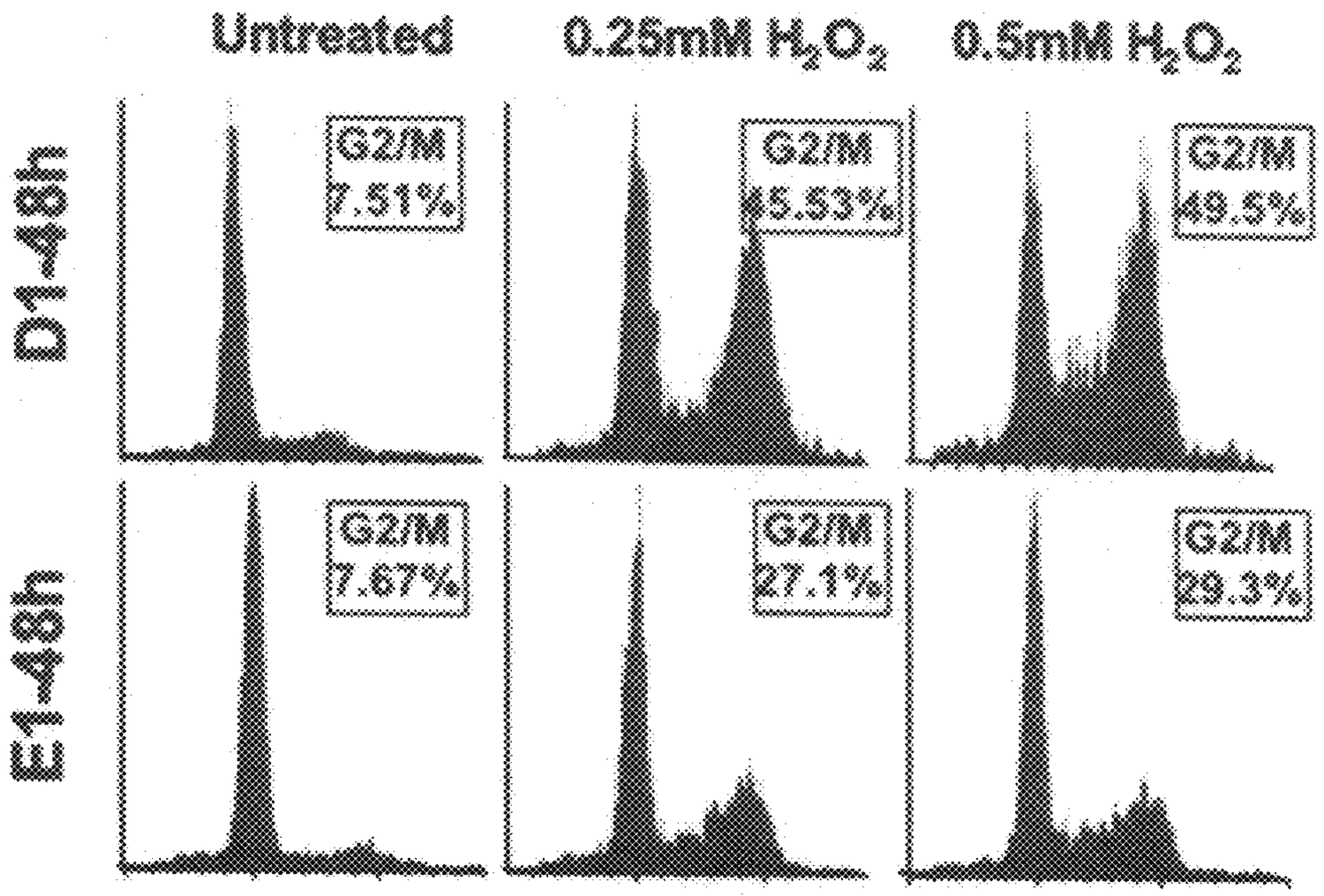


Figure 4E

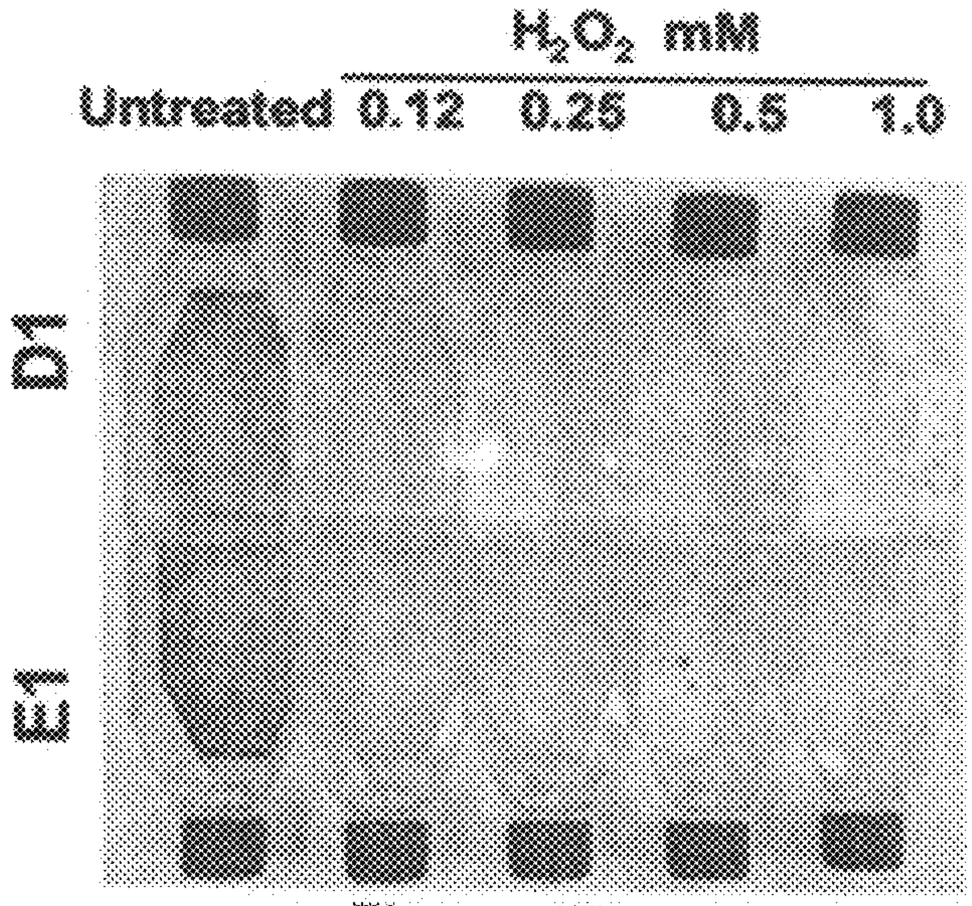


Figure 4F

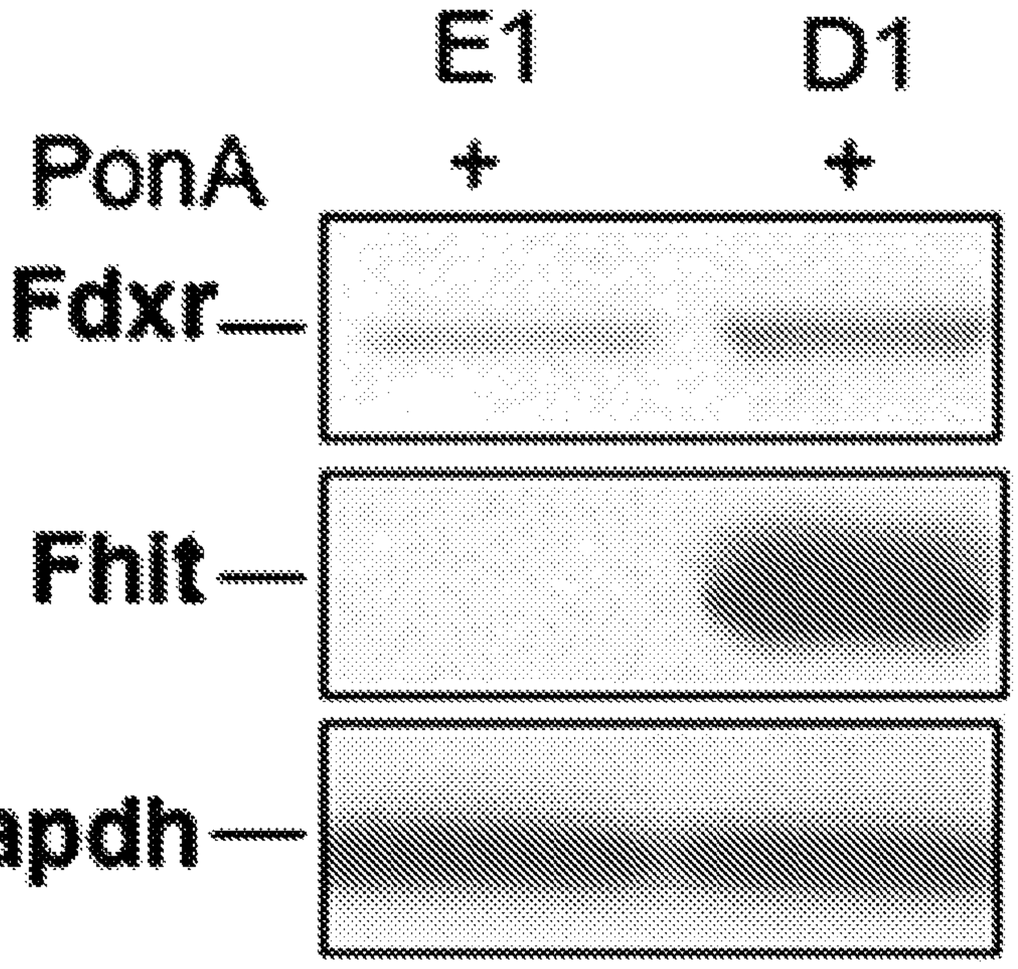


Figure 5A

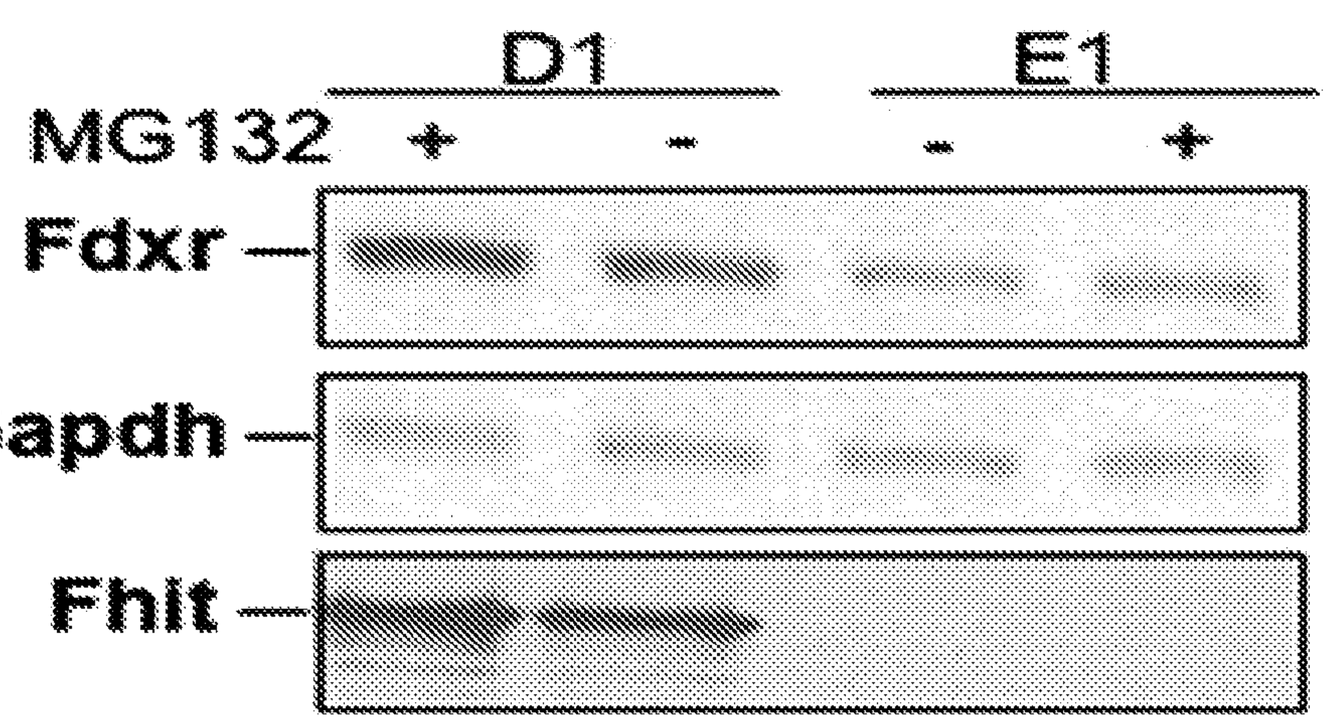


Figure 5B

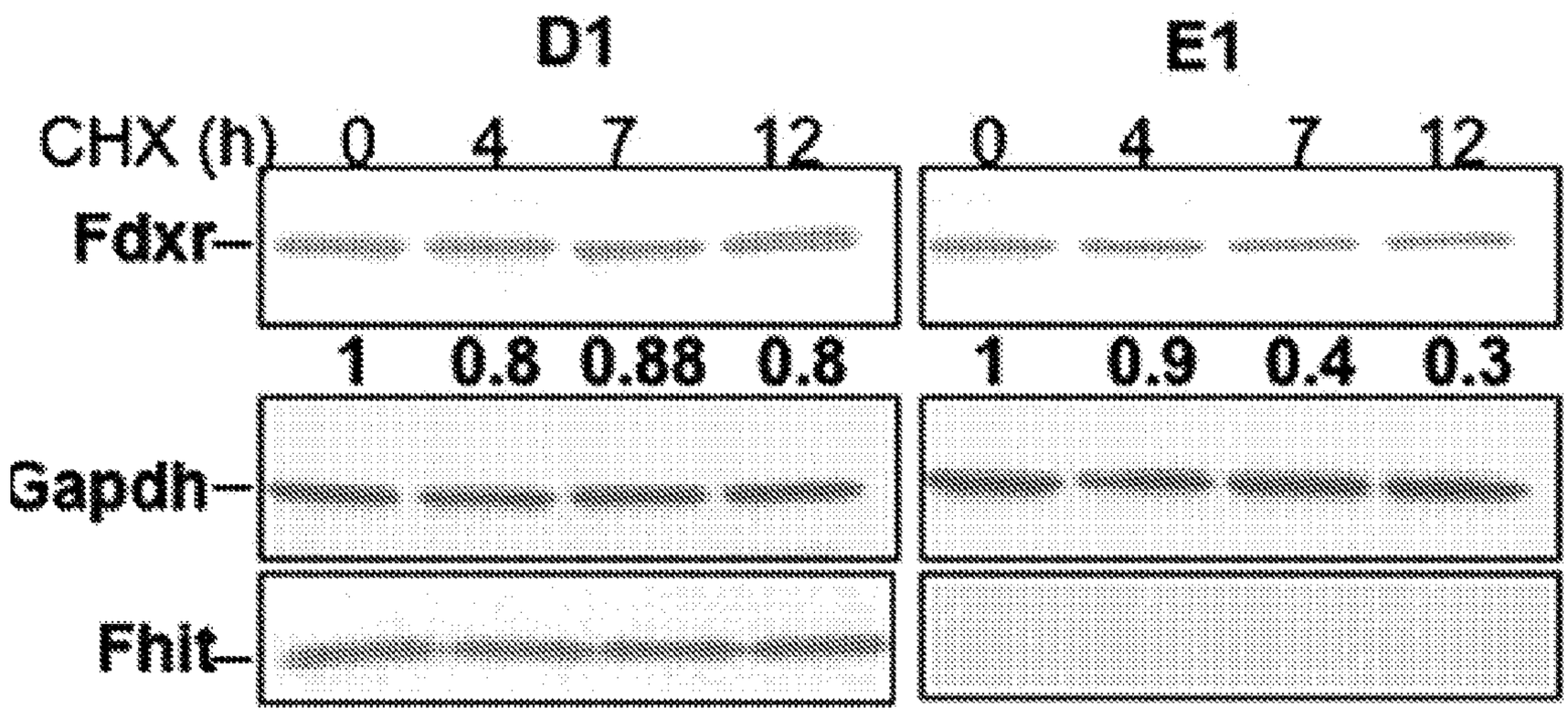


Figure 5C

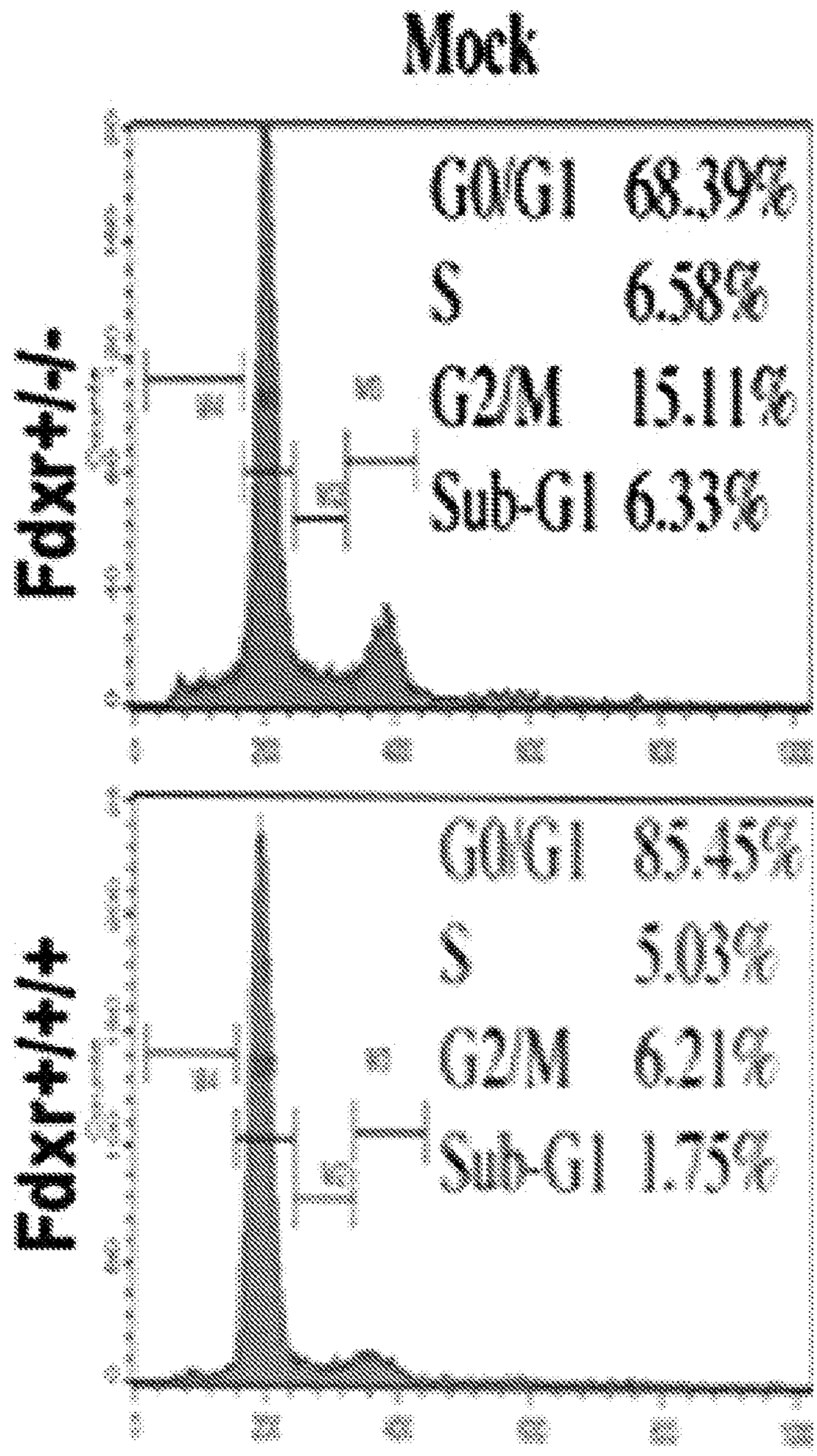

Mock
Fdxr+/+/+
G0/G1 68.39%
S 6.58%
G2/M 15.11%
Sub-G1 6.33%
Fdxr+/+/+
G0/G1 85.45%
S 5.03%
G2/M 6.21%
Sub-G1 1.75%

Figure 5D

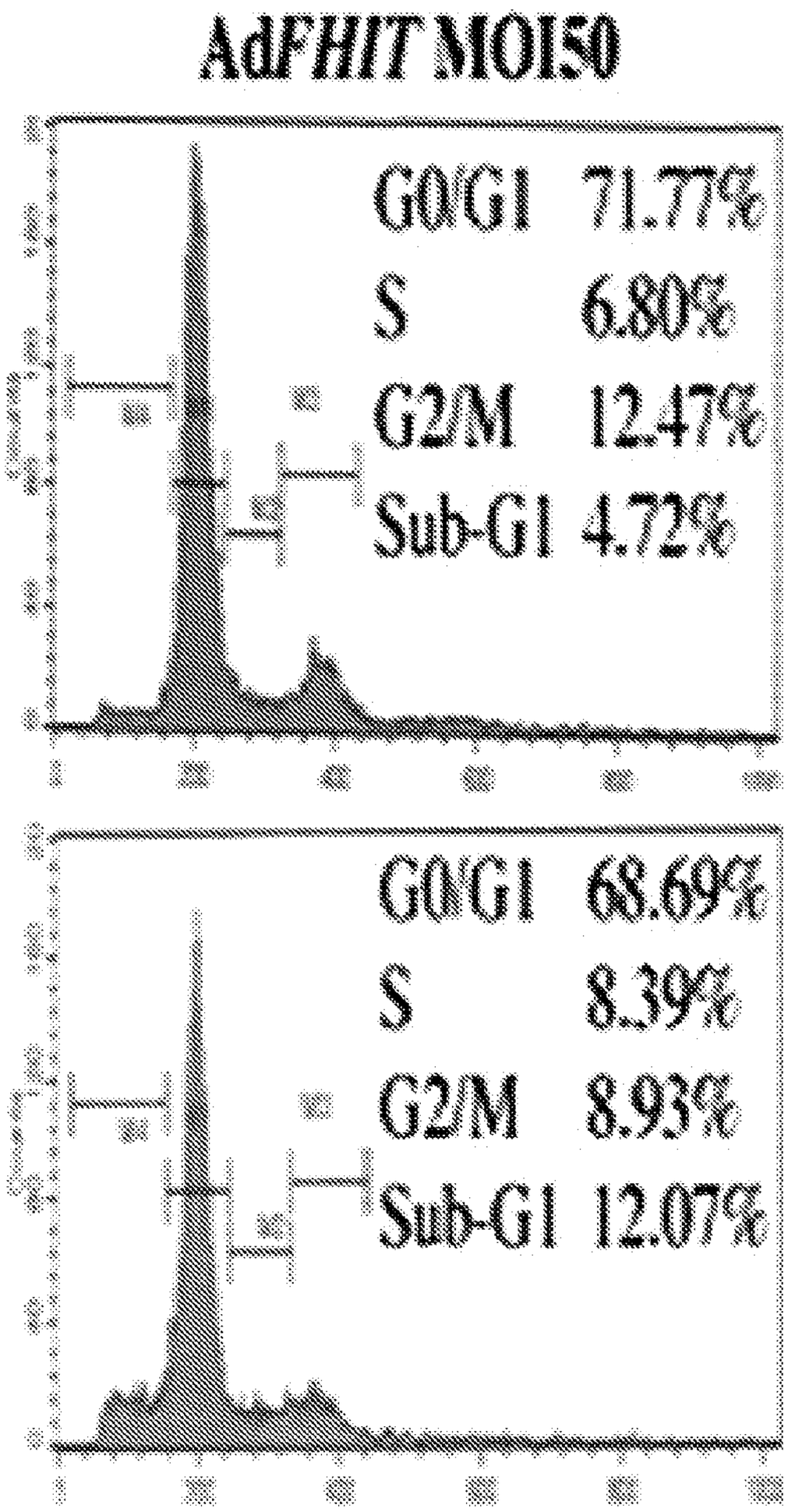
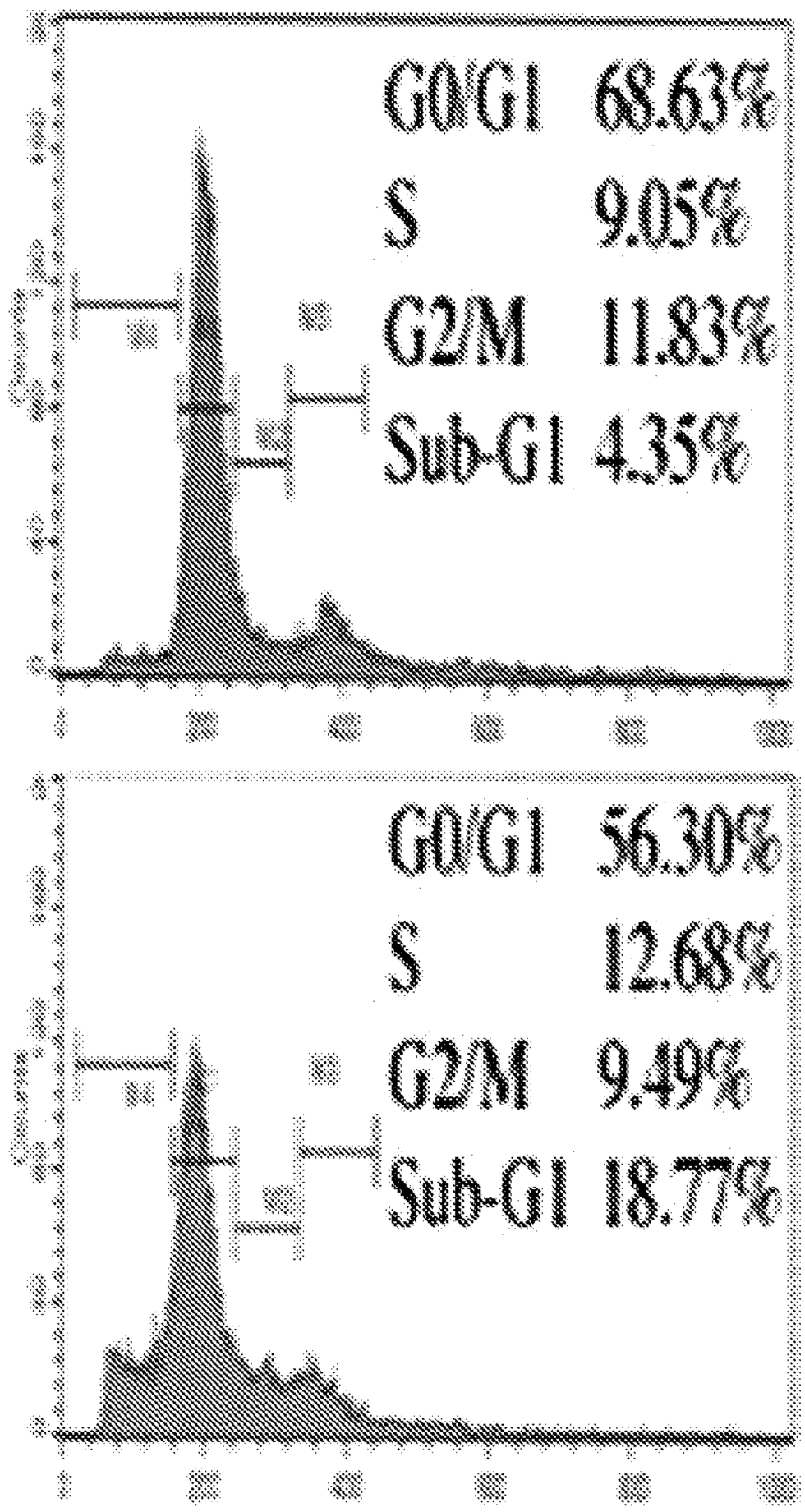


Figure 5D cont.



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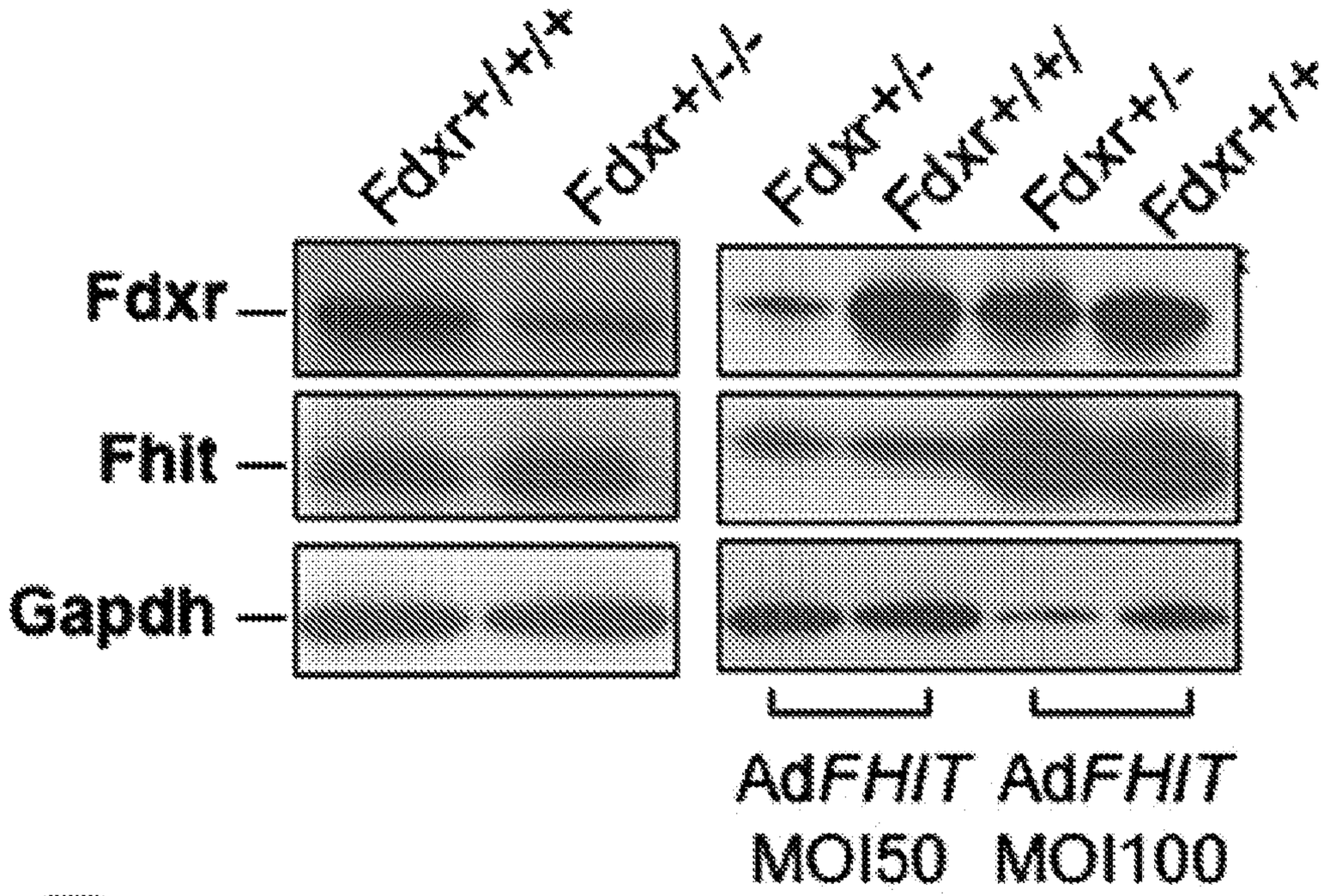


Figure 5E

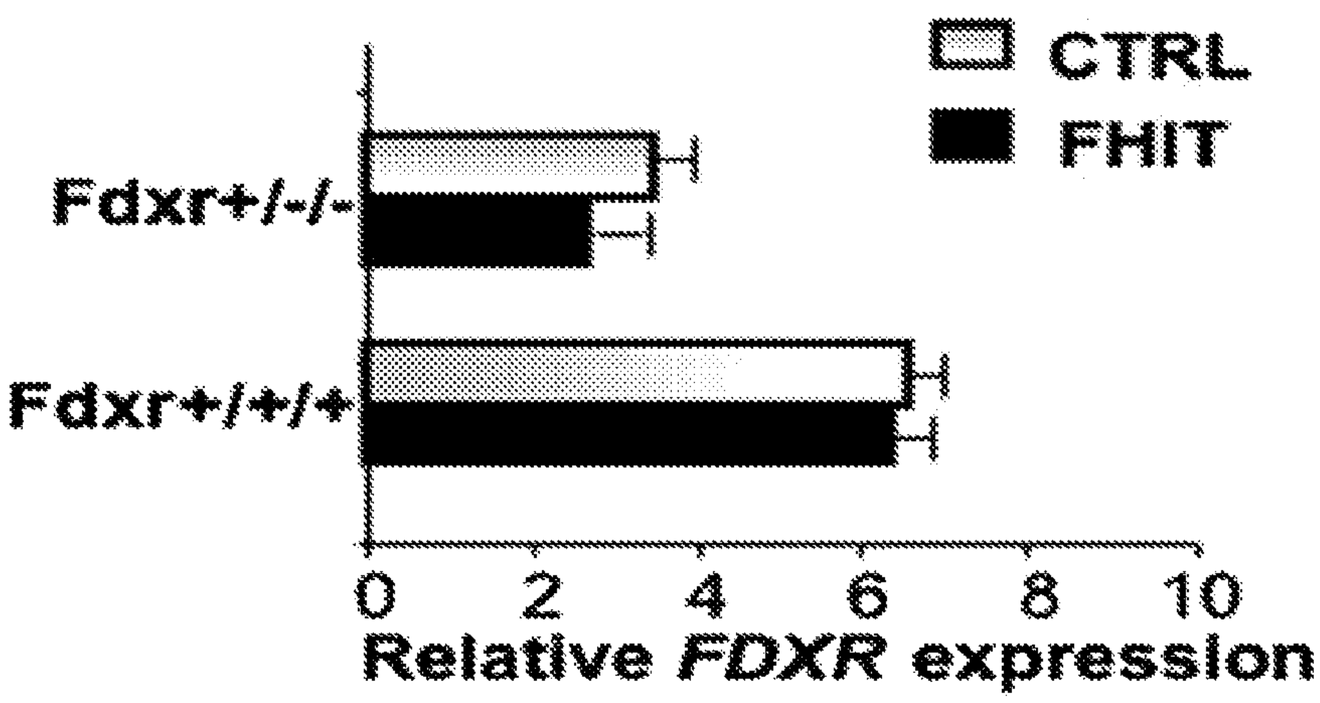


Figure 5F

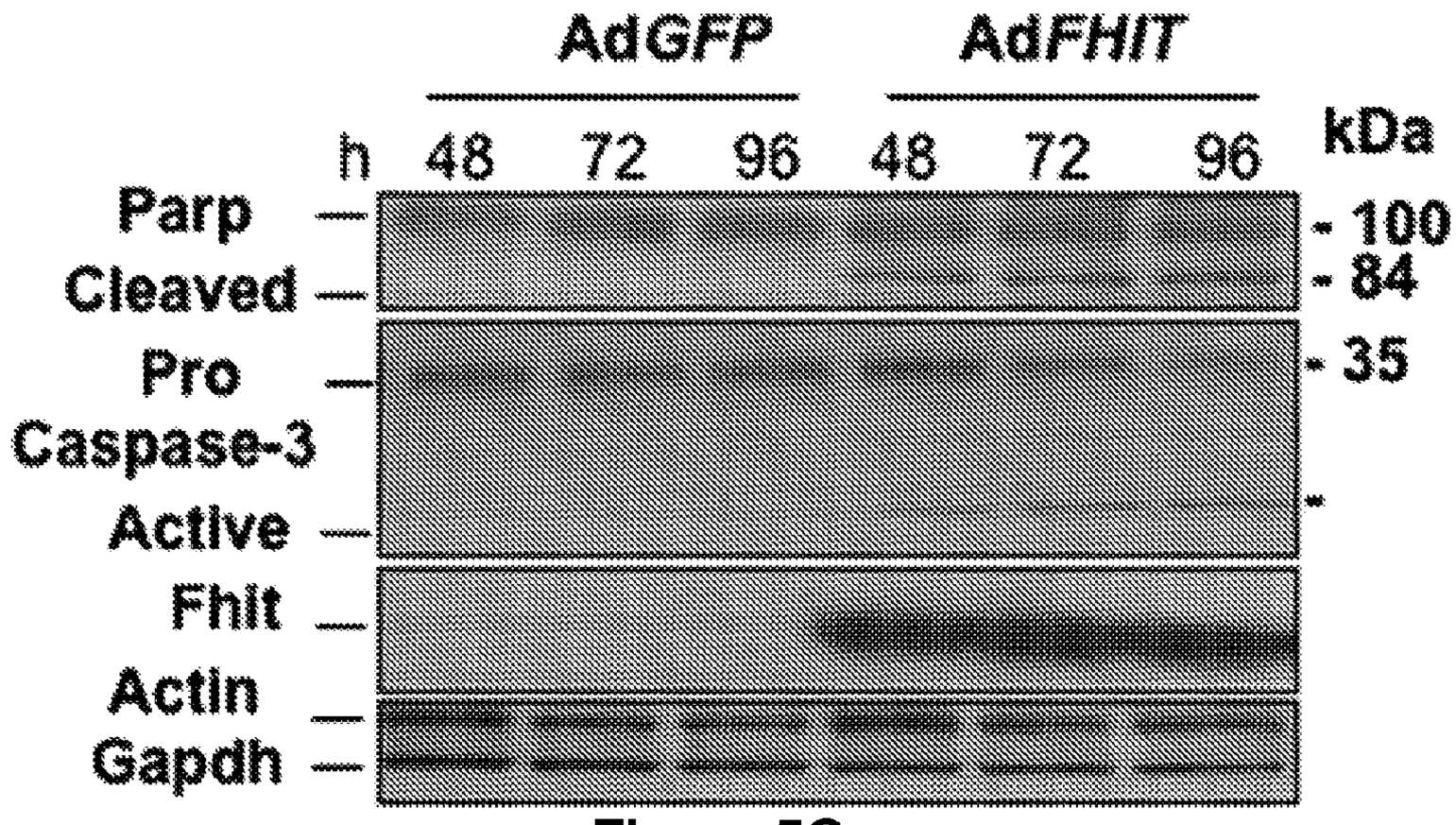


Figure 5G

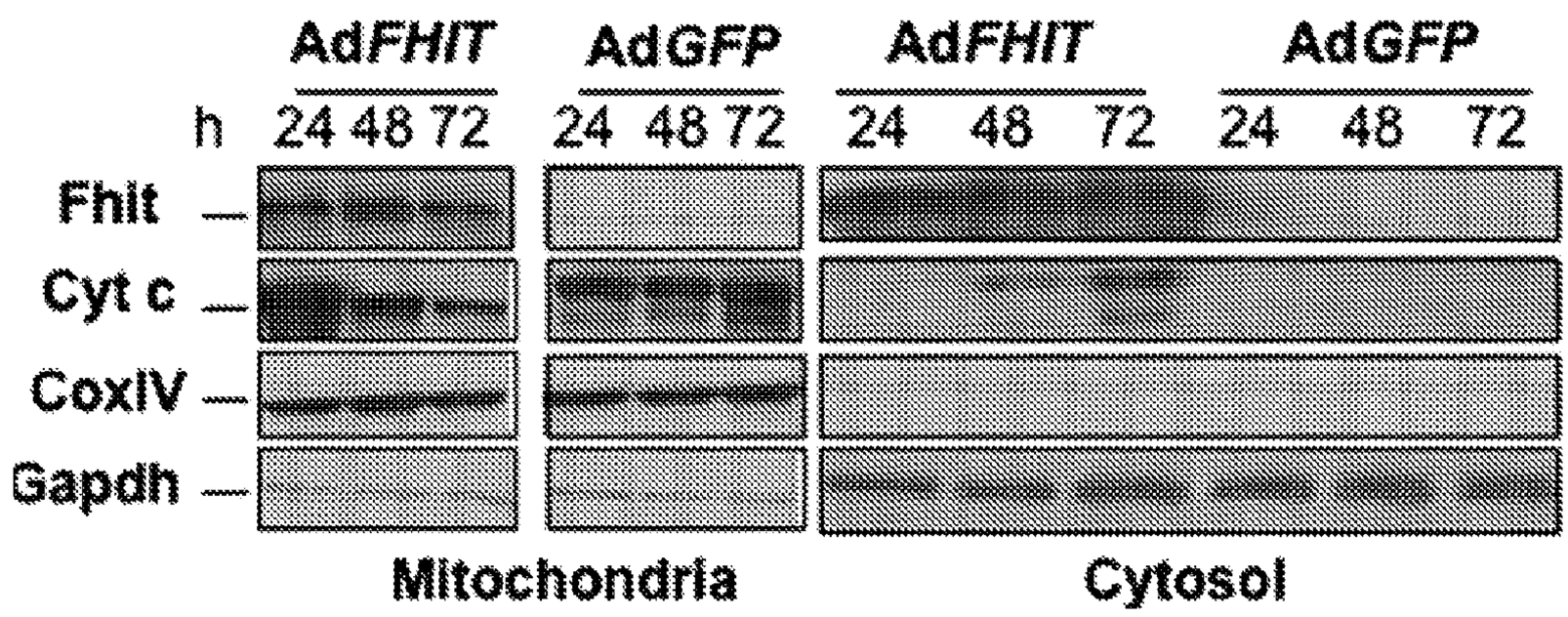
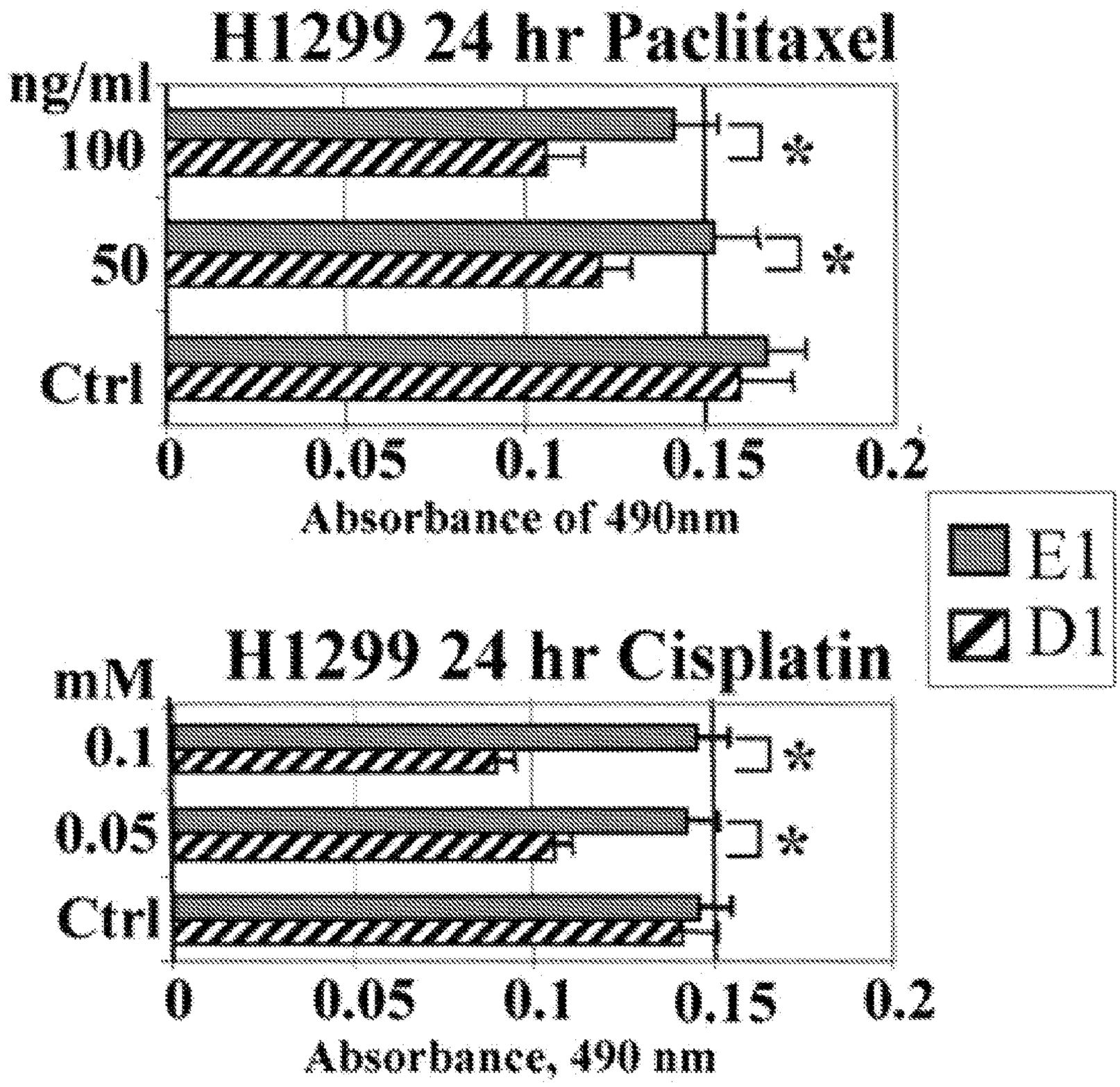
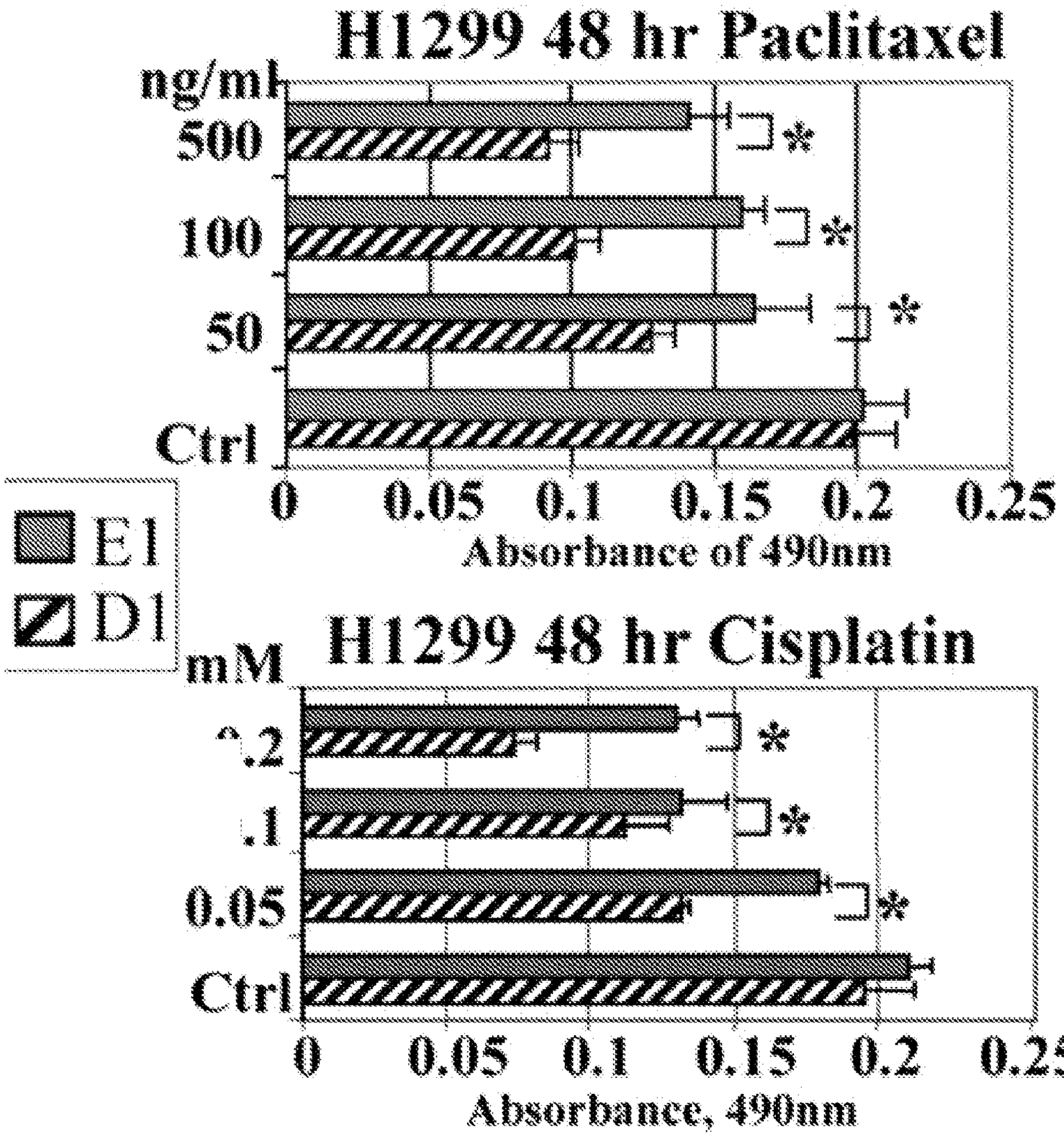


Figure 5H



Figures 6A and 6B



Figures 6A and 6B cont.

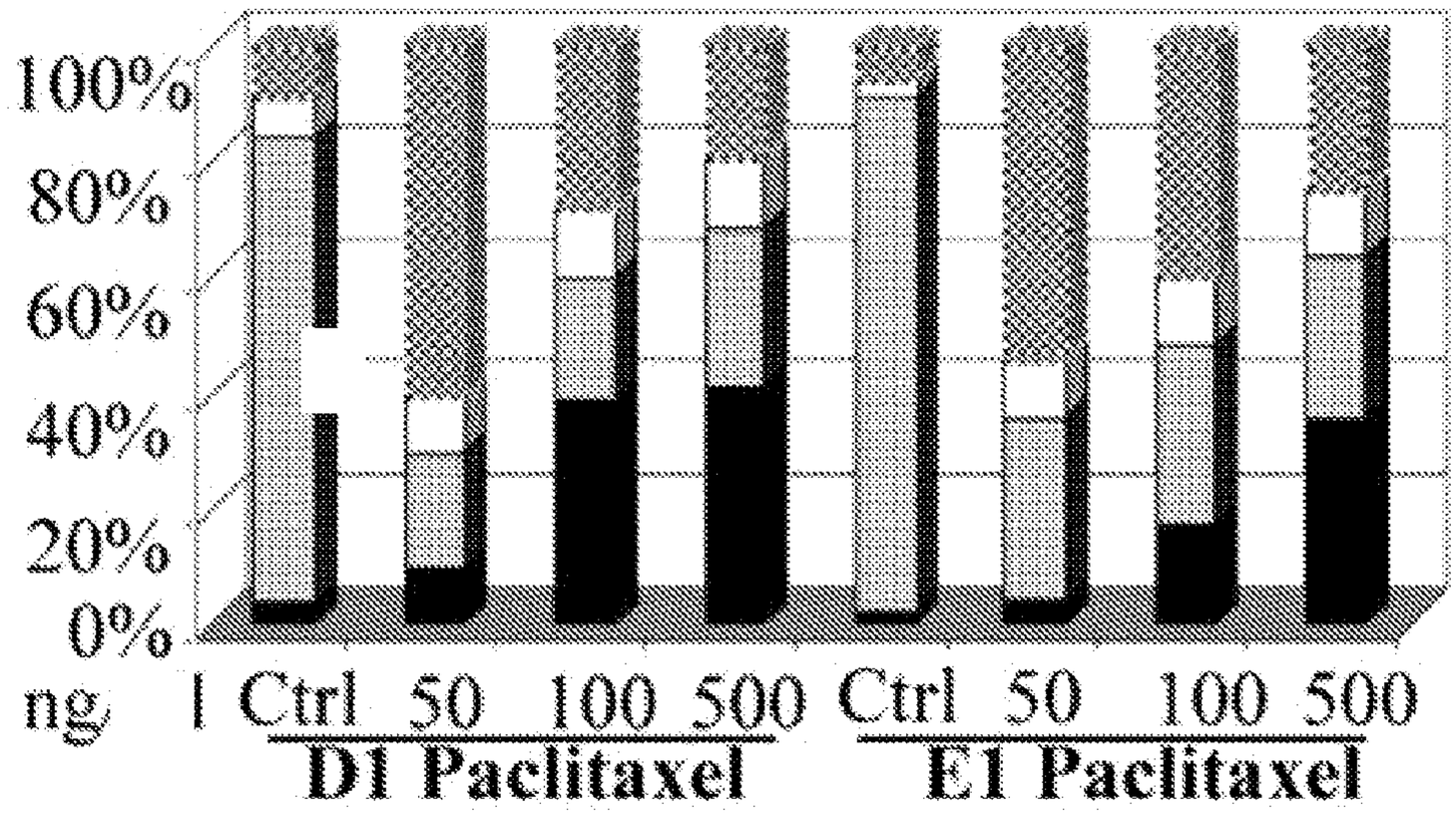


Figure 6C

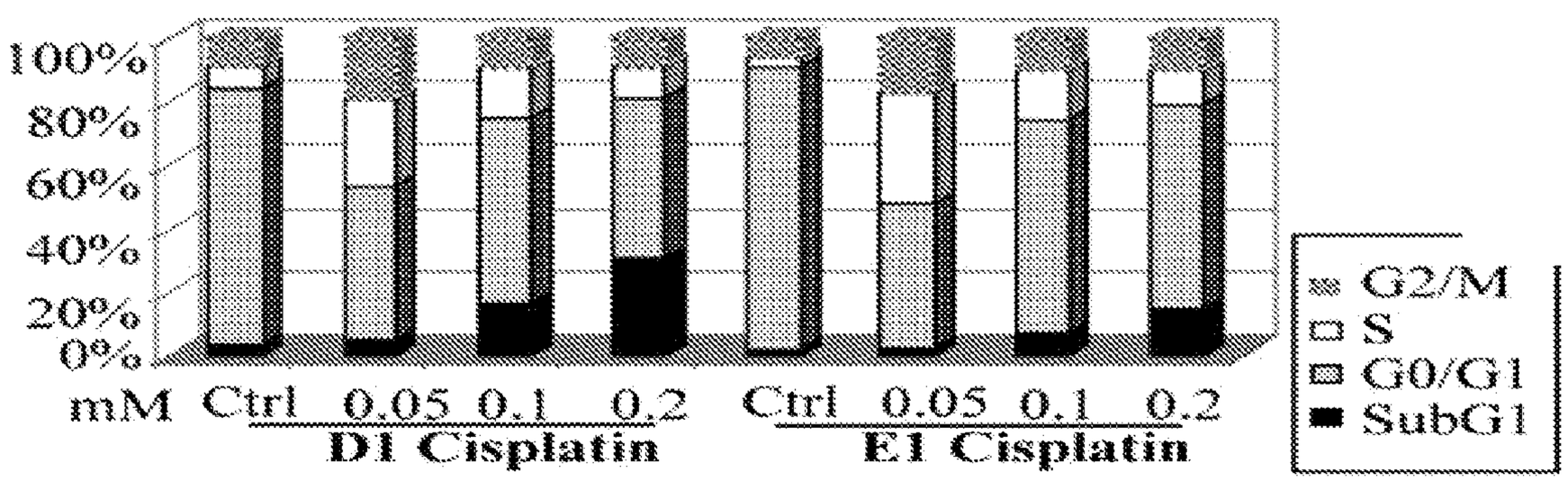


Figure 6D

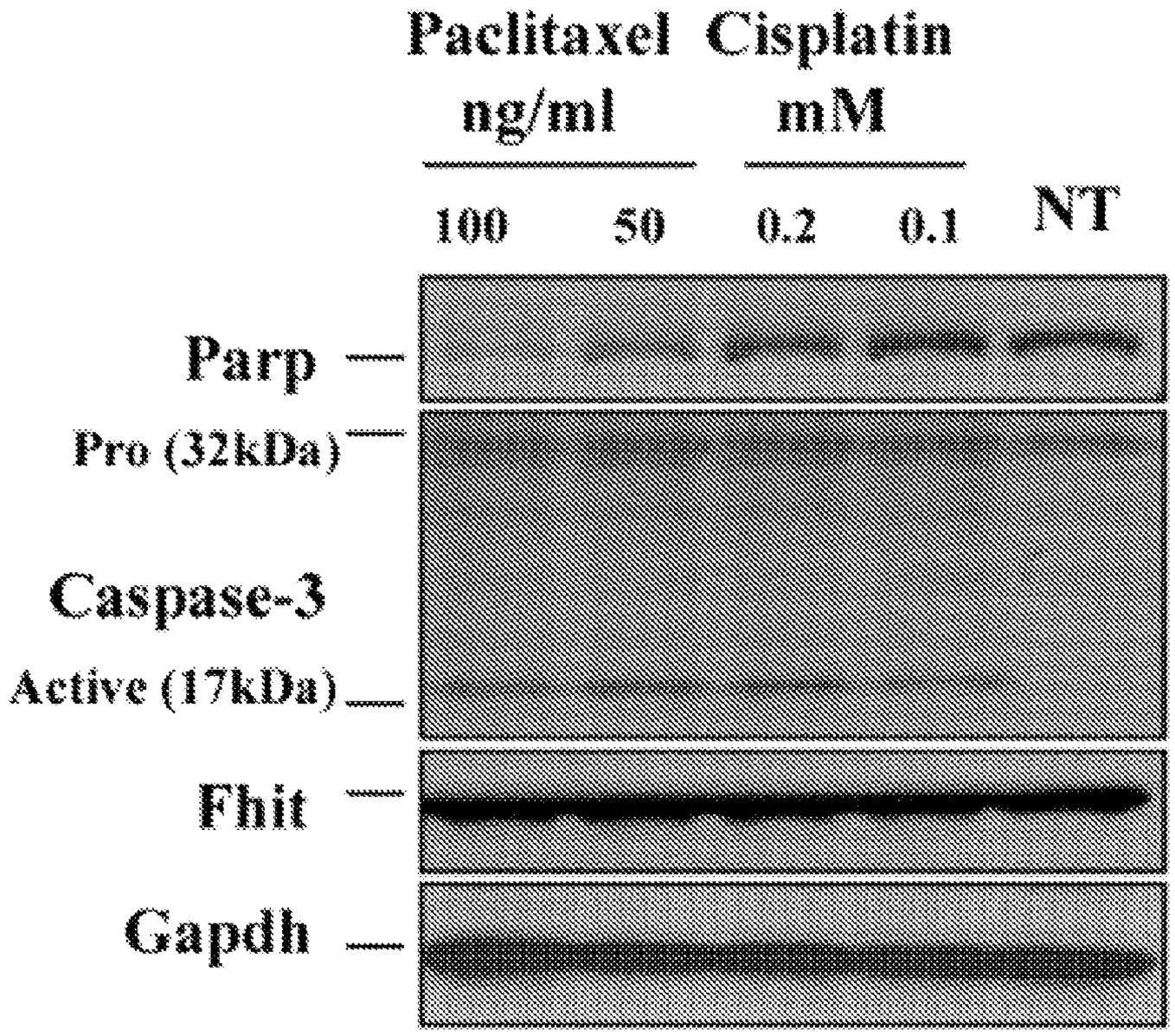

Paclitaxel Cisplatin
ng/ml
mM
100
50
0.2
0.1
NT
Parp
Pro (32kDa)
Caspase-3
Active (17kDa)
Fhit
Gapdh

Figure 6E

Protein	Accession no.	Molecular mass *kDa*	Function/ category	Subcellular localization	No. identified peptides	Peptide sequences	SEQ ID NO:	Protein Mascot score	Sequence coverage
Hsp60	NP_ 002147	60	60-kDa Heat shock protein	Cytosol/ mitochondria	6	VGEVIVTK	**1**	239	10%
						LSDGVAVLK	**2**		
						IGIEIIKR	**3**		
						VTDALNATR	**4**		
						TVIIEQSWGSPK	**5**		
						VGGTSDVEVNEKK	**6**		
Malate dehydro-genase (Mdh)	NP_ 005909	33	Catalyzes the reversible oxidation of malate to oxaloacetate	Mitochondrial matrix	8	ANTFVAELK	**7**	193	28%
						IQEAGTEVVK	**8**		
						VNVPVIGGHAGK	**9**		
						IFGVTTLDIVR	**10**		
						FVFSLVDAMNGK	**11**		
						GCDVVVIPAGVPR	**12**		
						AGAGSATLSMAYAGAR	**13**		
						GYLGPEQLPDCLK	**14**		
Electron transfer flavor protein (Etfb)	NP_ 001976	28	Specific electron acceptor for mitochondrial dehydro-genases	Mitochondrial matrix	3	EIDGGLETLR	**15**	96	12%
						VETTEDLVAK	**16**		
						LSVISVEDPPQR	**17**		
Hsp10	AAC 96332	10	10-kDa Heat shock protein	Cytosol/mitochondria	3	GGEIQPVSVK	**18**	92	34%
						VLQATVVAVGSGSK	**19**		
						VVLDDKDYFLFR	**20**		
Mitochon-drial aldehyde dehydro-genase 2 (Adh2)	NP_ 000681	55	Second enzyme of the major oxidative pathway of alcohol metabolism	Mitochondrial matrix	2	LADLIER	**21**	75	4%
						LGPALATGNVVVMK	**22**		
Ferre-doxin reductase (Fdxr)	P22570	54	First electron transfer protein in all the mitochondrial p450 systems	Mitochondrial matrix	1	FGVAPDHPEVK	**23**	47	2%

Figure 7 - TABLE 1

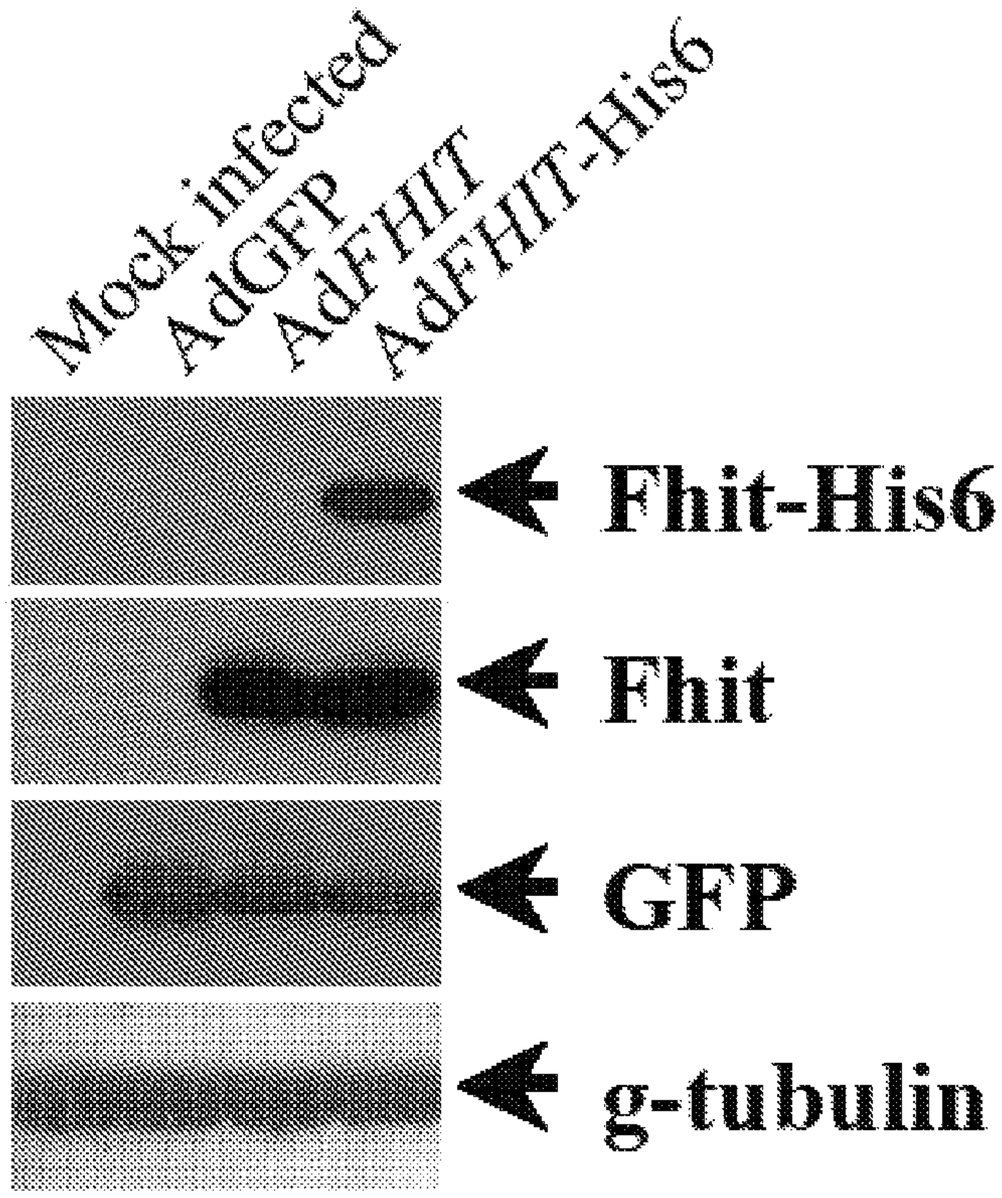
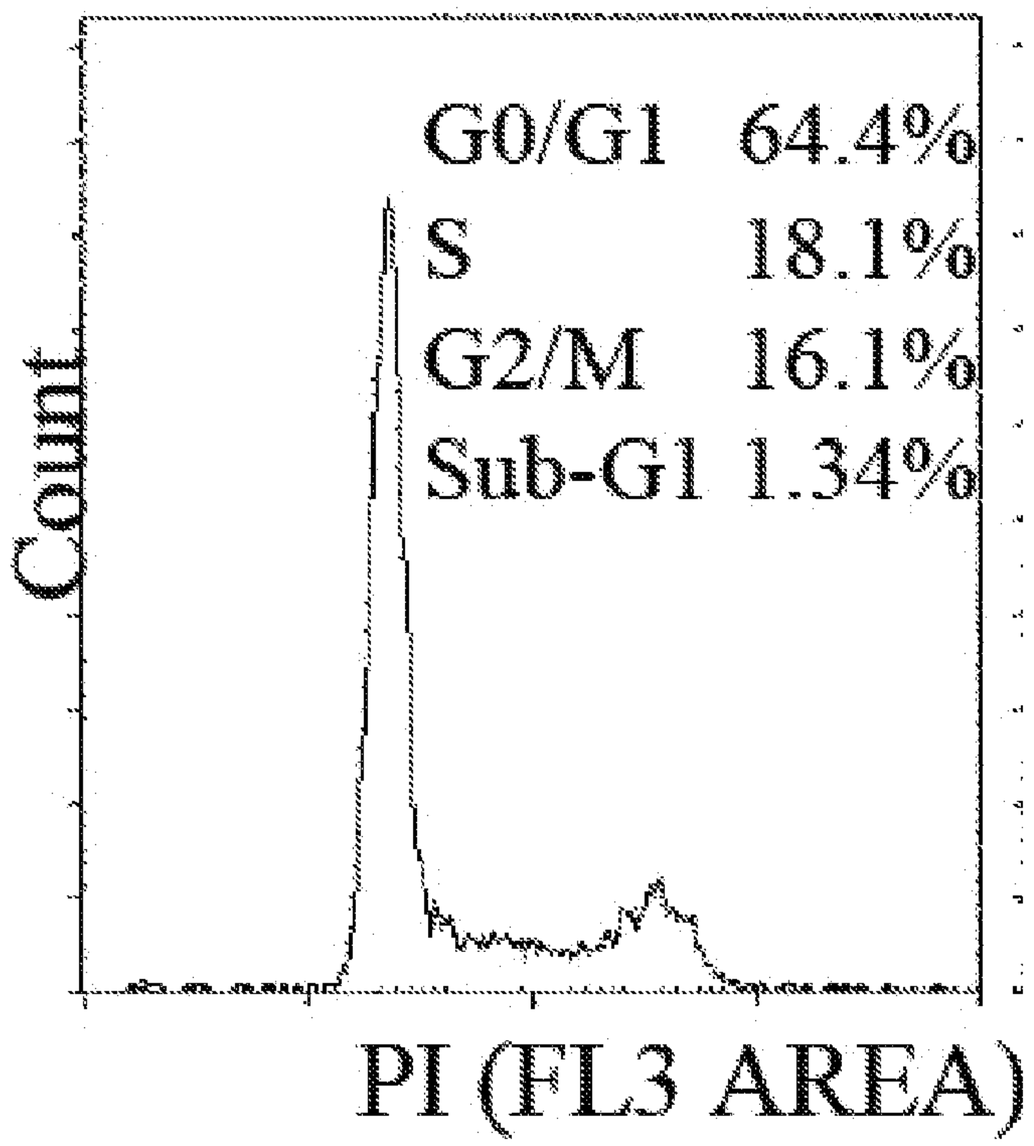

Mock infected
AdGFP
AdFHIT
AdFHIT-His6
Fhit-His6
Fhit
GFP
g-tubulin

Figure 8A


Mock infected
G0/G1 64.4%
S 18.1%
G2/M 16.1%
Sub-G1 1.34%
Count
PI (FL3 AREA)

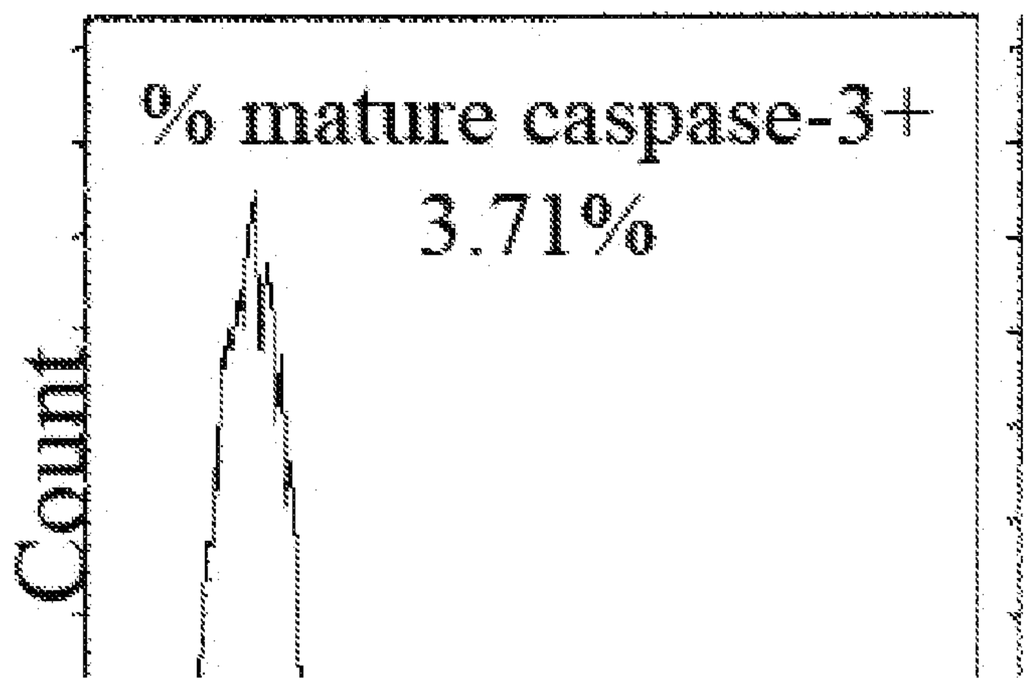

% mature caspase-3+
3.71%
Count

Figure 8B

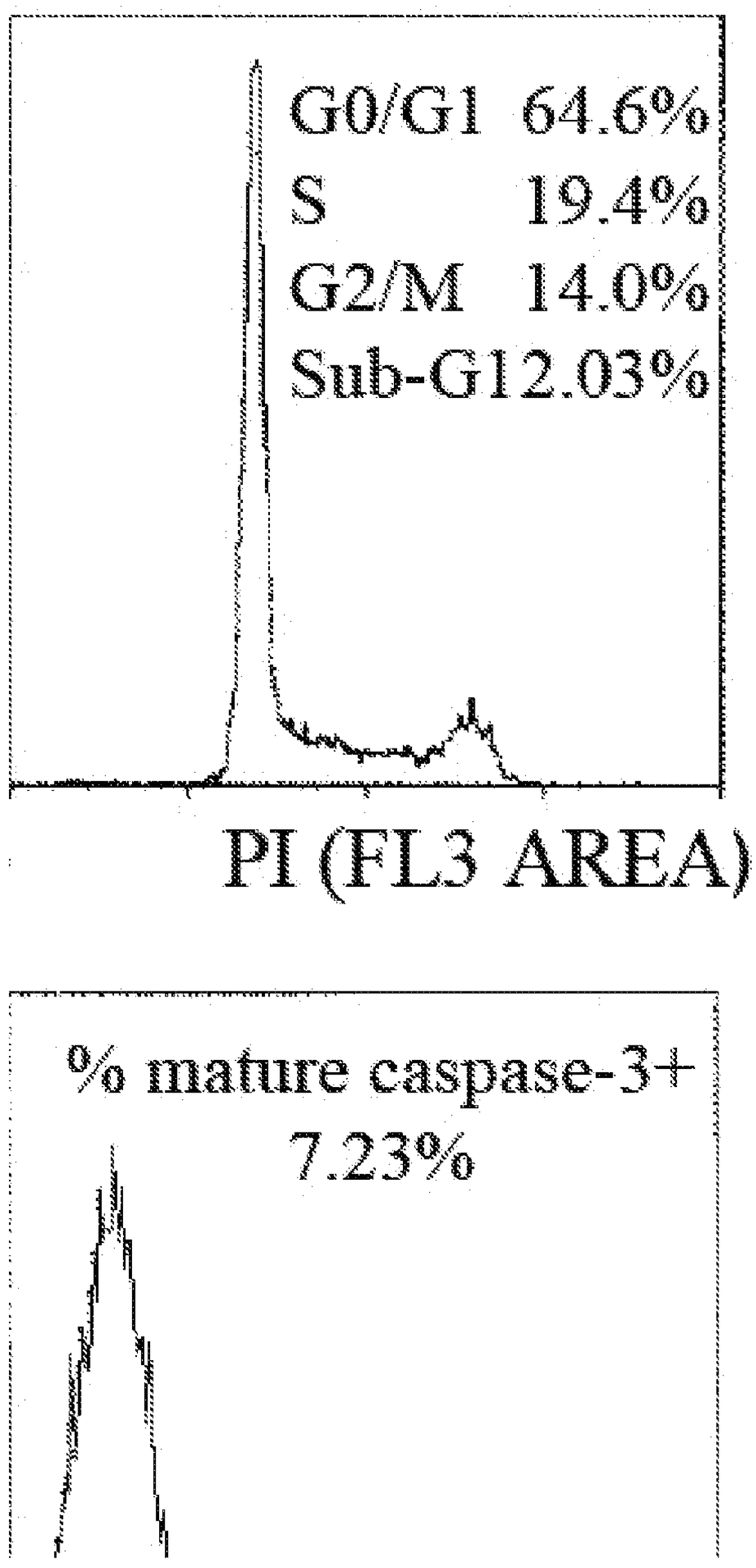


Figure 8B cont.

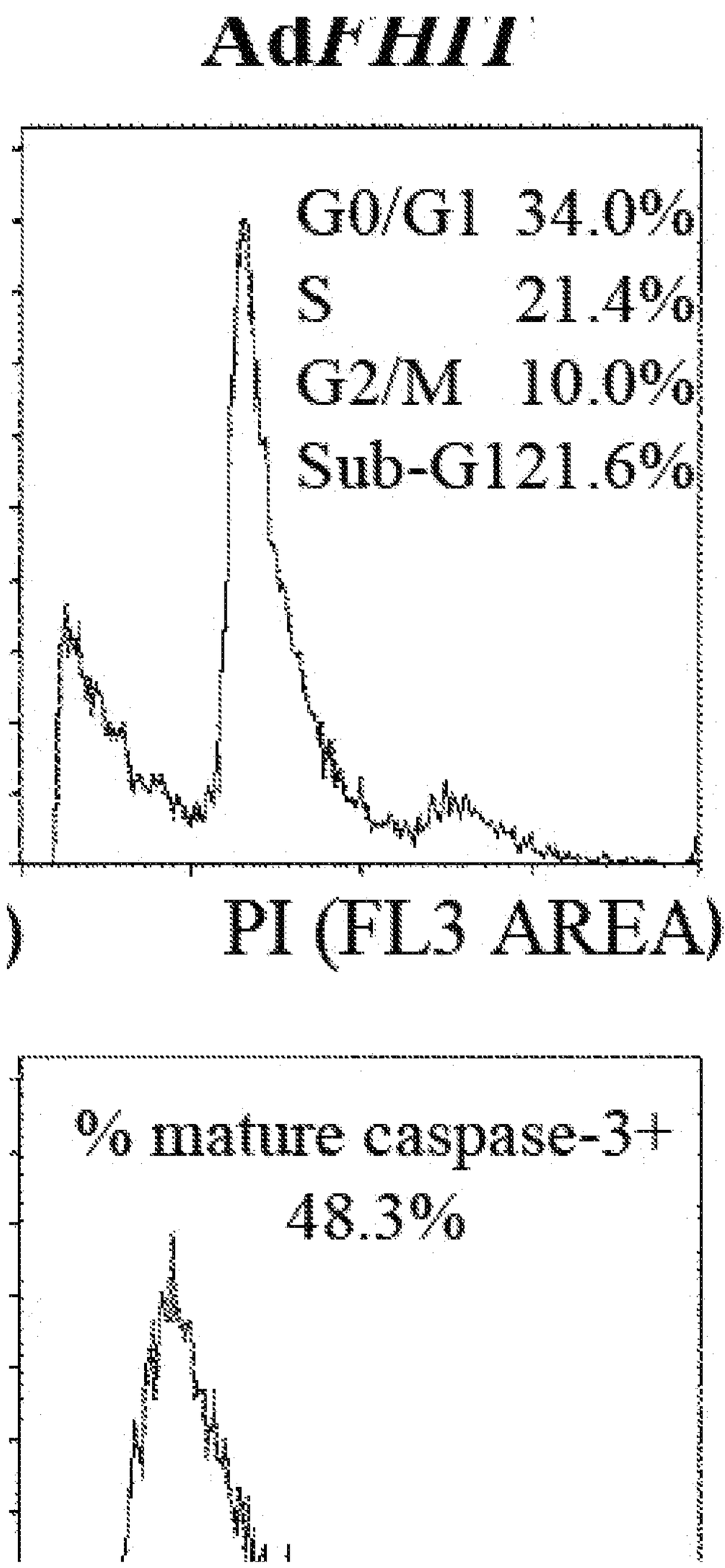


Figure 8B cont

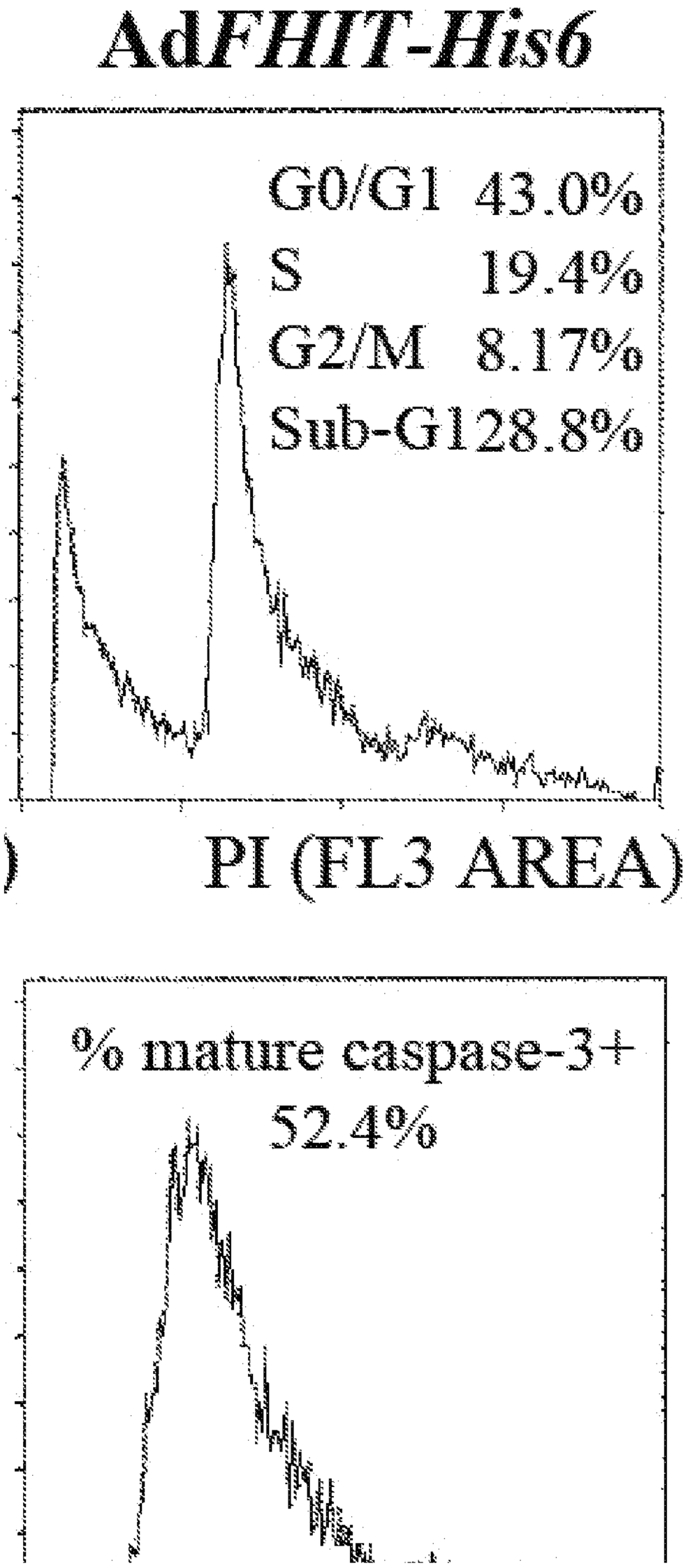
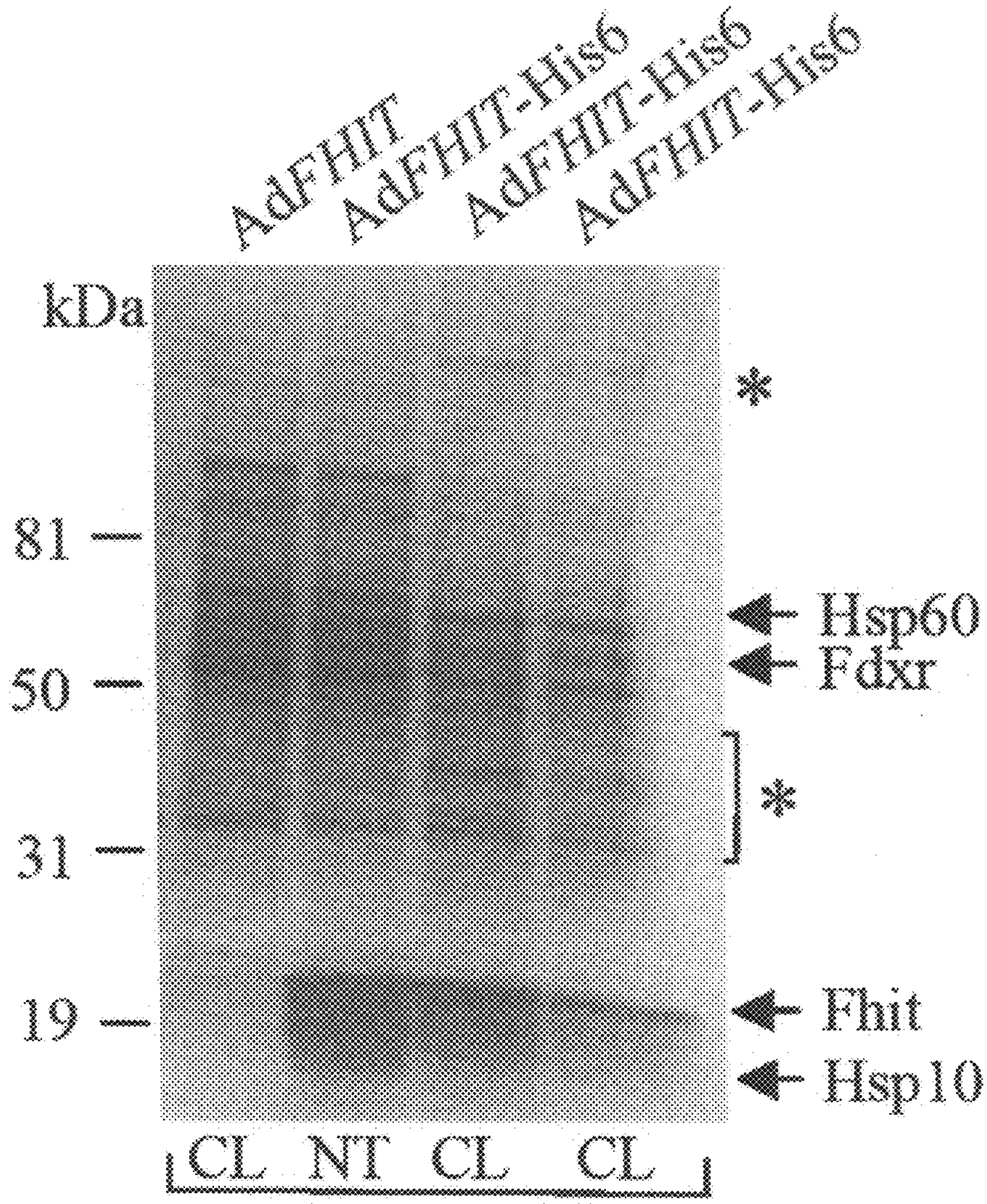
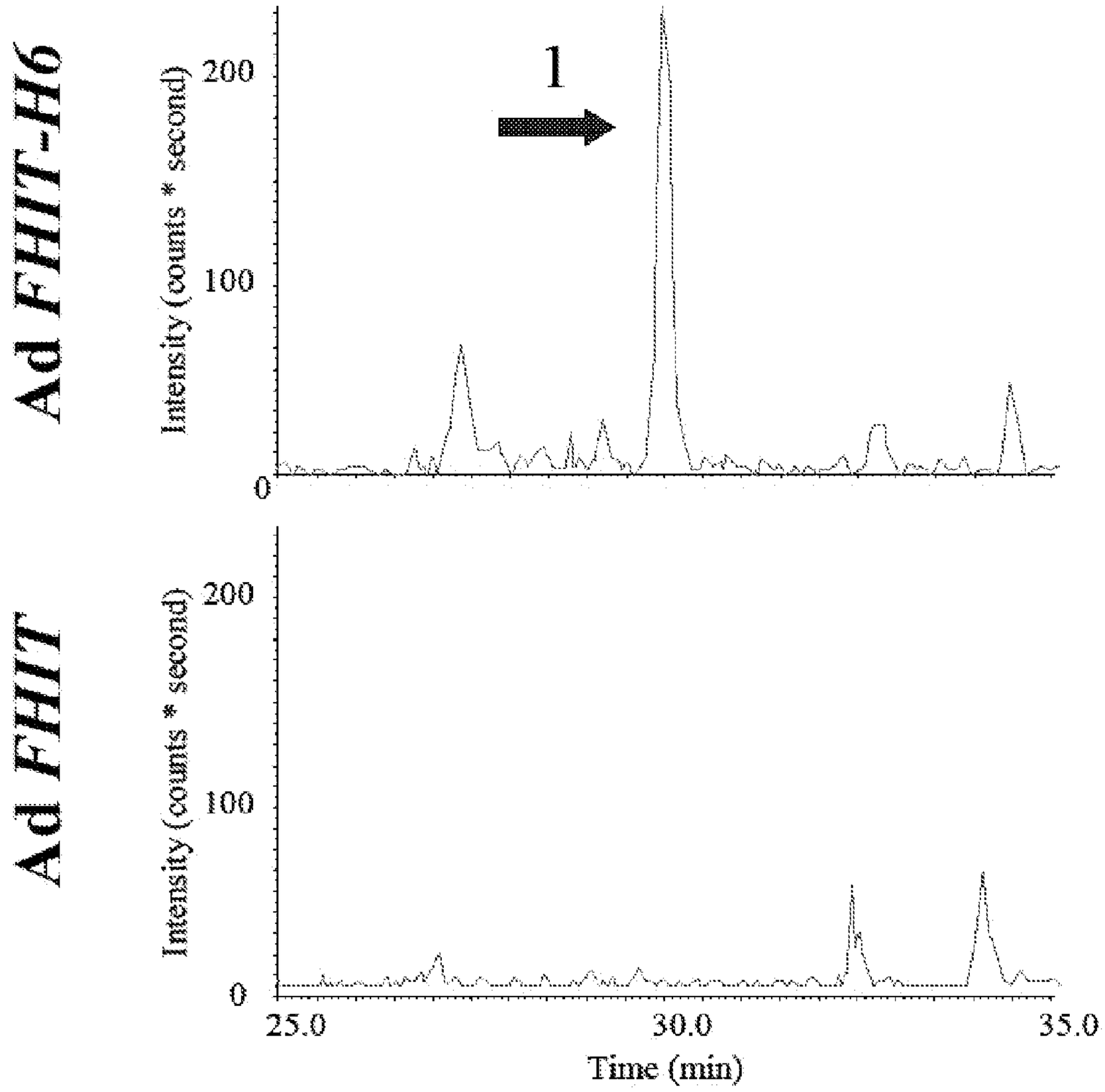


Figure 8B cont.



His6-Nickel pull down

Figure 8C



Hsp60

Figure 9A

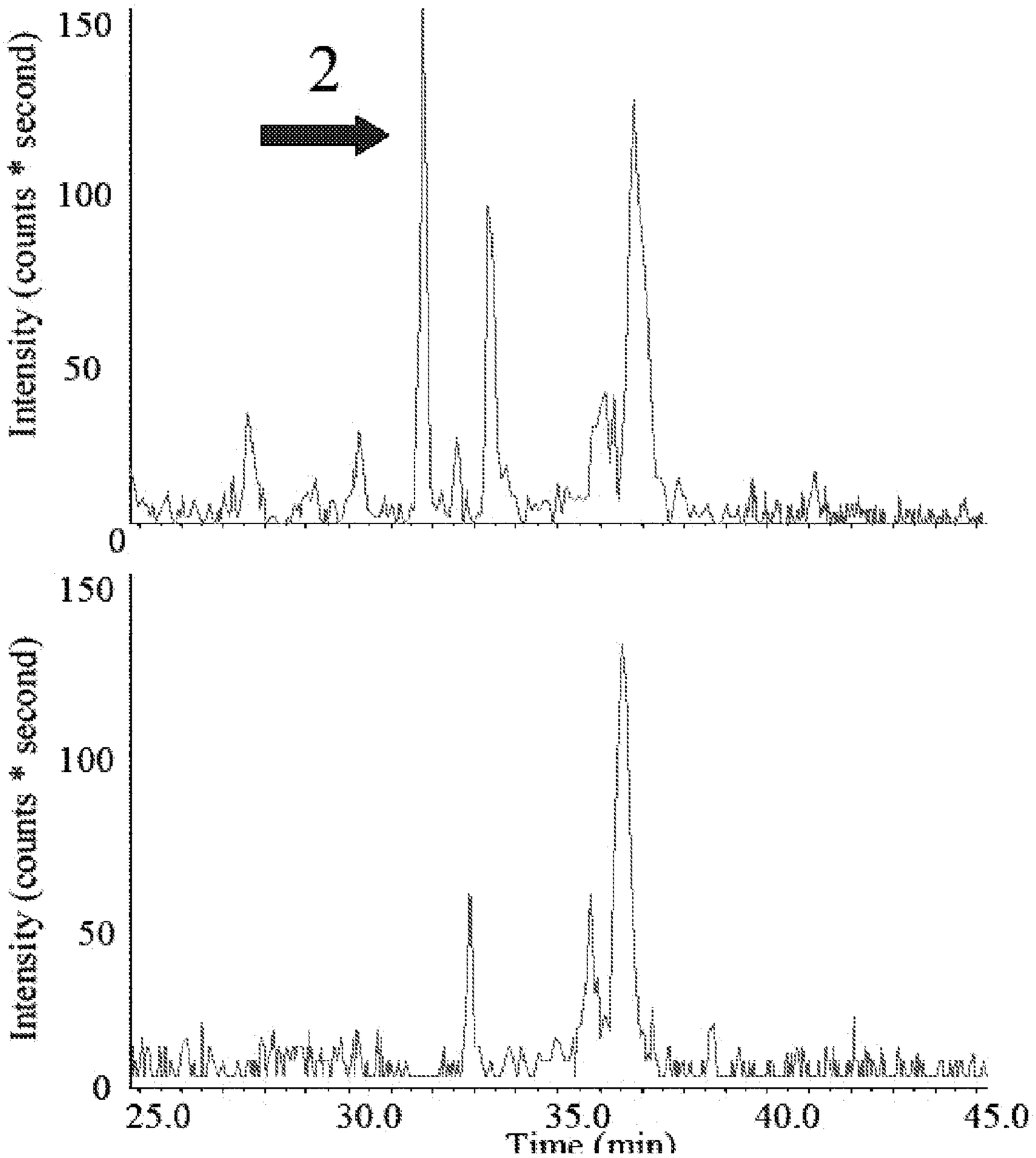

2
Intensity (counts * second)
150
100
50
0
Intensity (counts * second)
150
100
50
0
25.0
30.0
35.0
40.0
45.0
Time (min)

Figure 9B

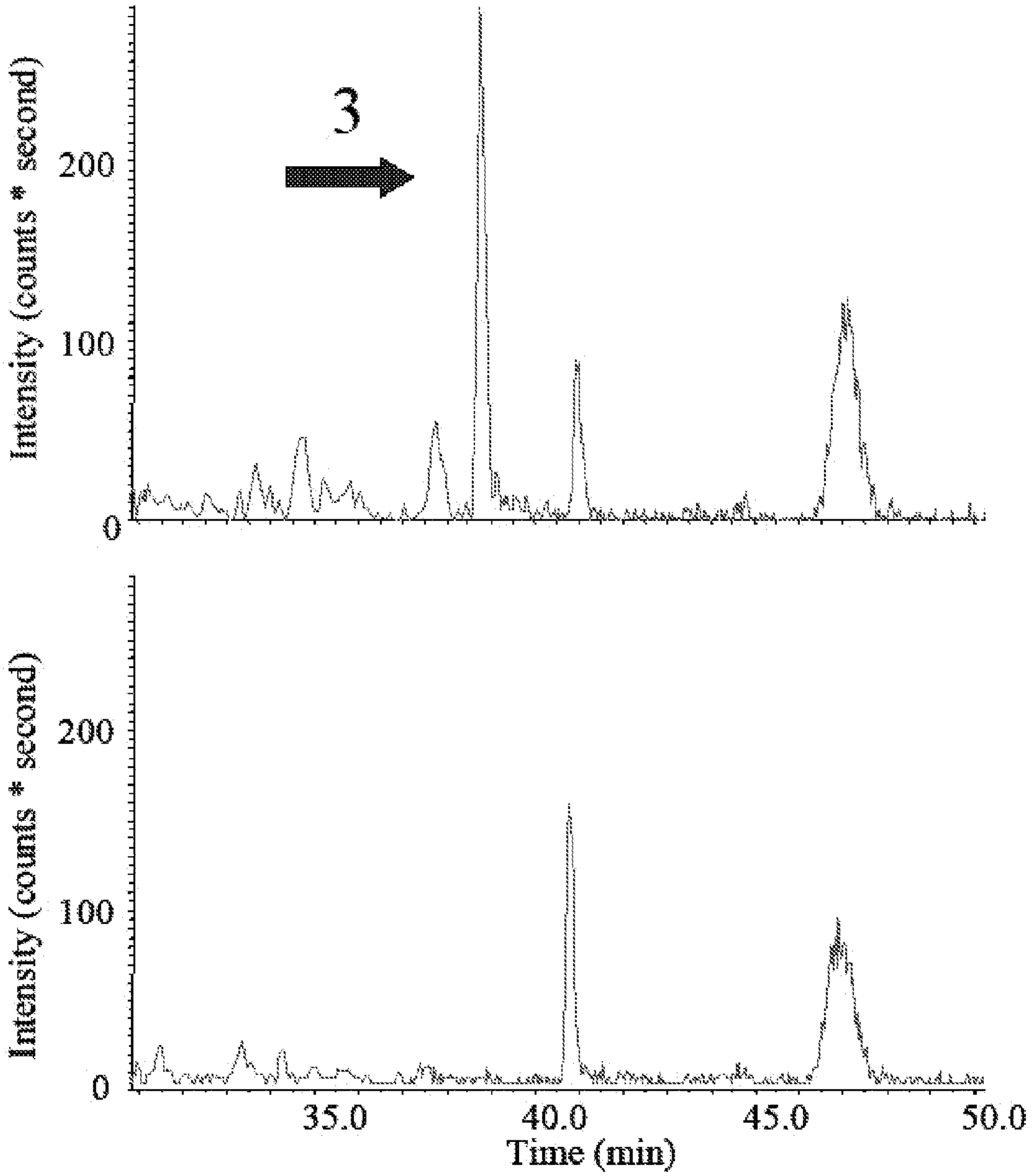

3
Intensity (counts * second)
200
100
0
Intensity (counts * second)
200
100
0
35.0
40.0
45.0
50.0
Time (min)

Figure 9C

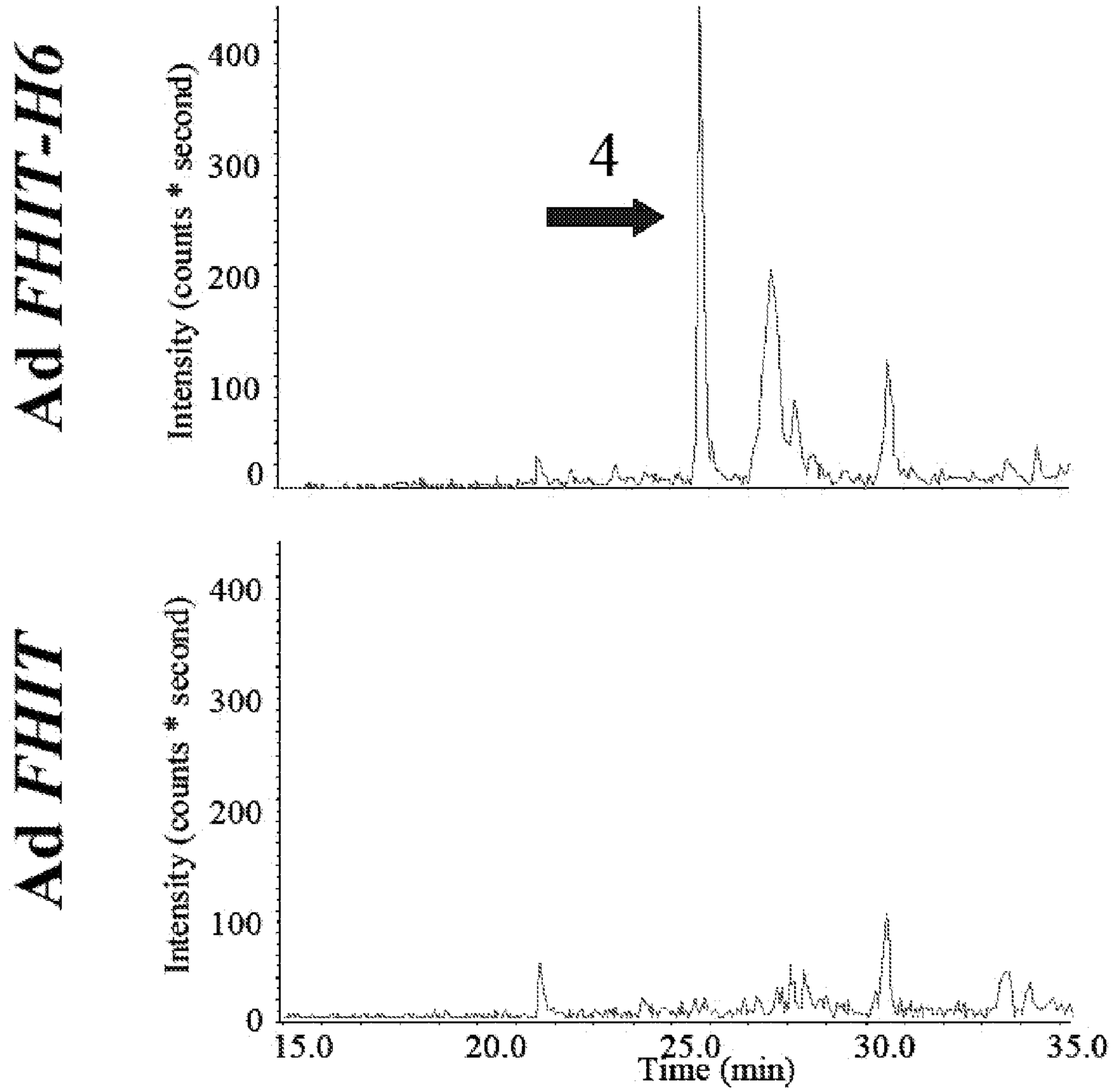

Ad FHIT-H6
Intensity (counts * second)
400
300
200
100
0
4
Ad FHIT
Intensity (counts * second)
400
300
200
100
0
15.0
20.0
25.0
30.0
35.0
Time (min)

Figure 9D

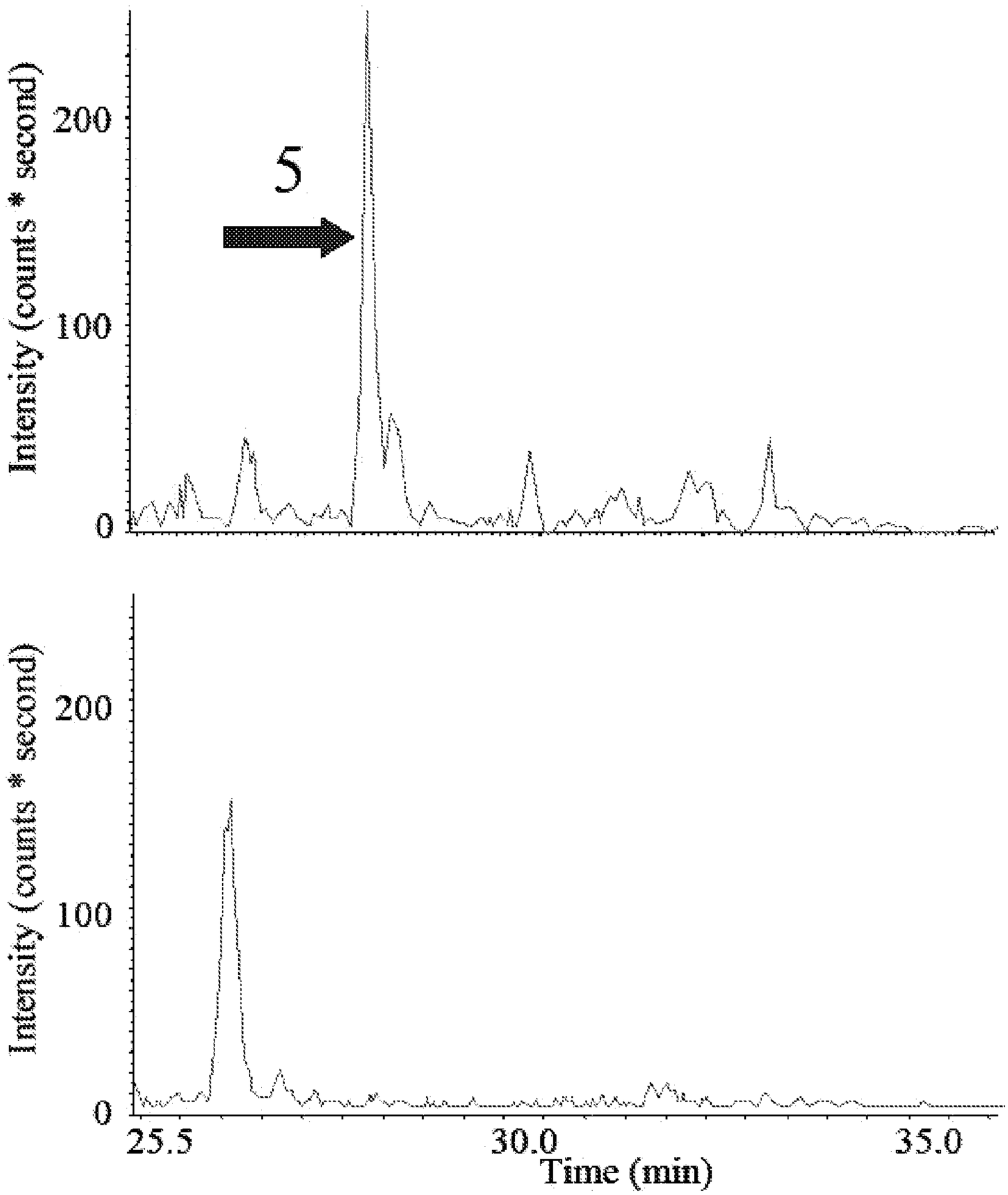

5
Intensity (counts * second)
200
100
0
Intensity (counts * second)
200
100
0
25.5
30.0
35.0
Time (min)

Figure 9E

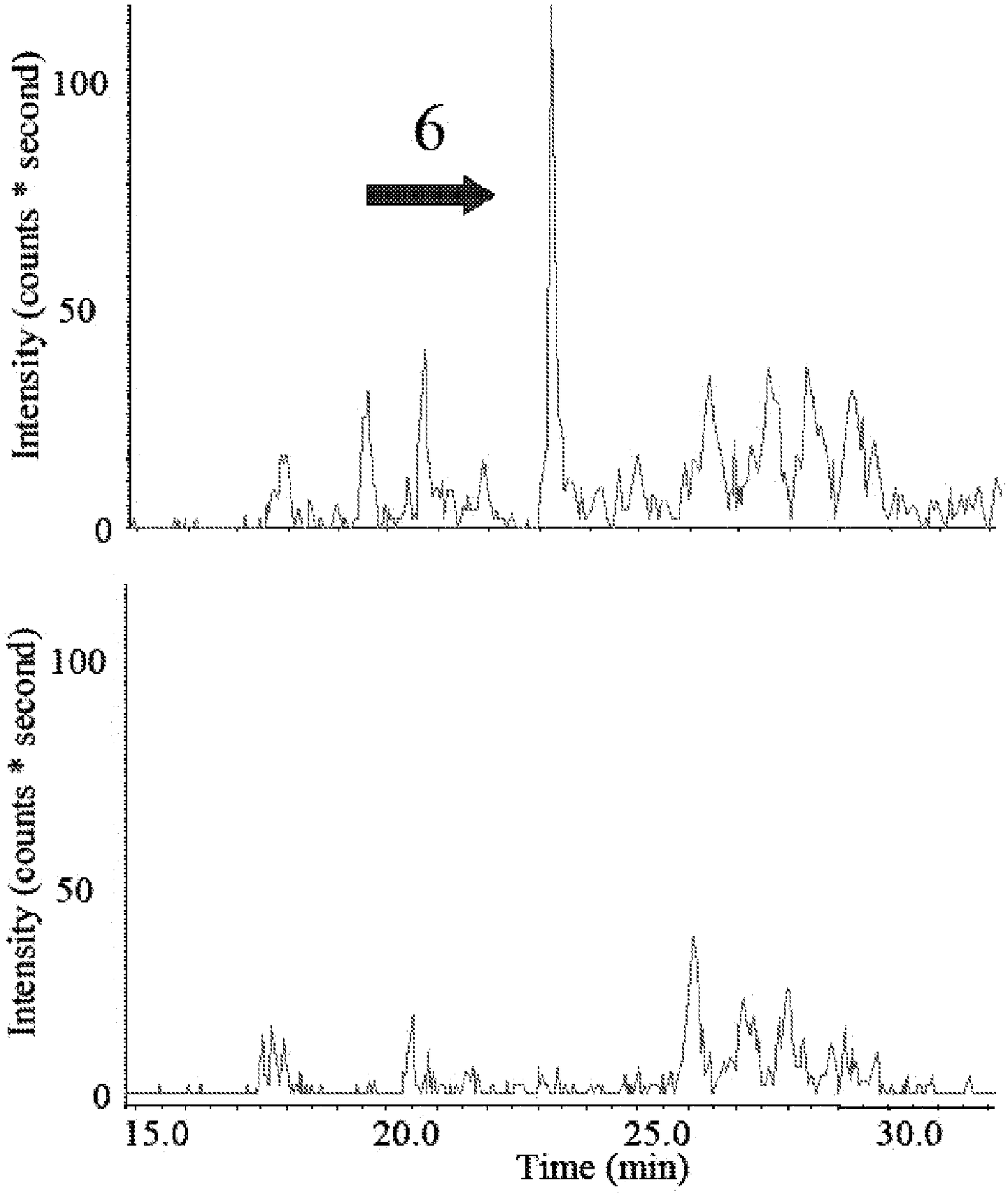

6
Intensity (counts * second)
100
50
0
Intensity (counts * second)
100
50
0
15.0
20.0
25.0
30.0
Time (min)

Figure 9F

	MKN74E4 (Fhit-)	**MKN74A116 (Fhit+)**
H_2O_2 (mM)	ROS-positive cells (%)	ROS-positive cells (%)
-	1.0	2.4
0.5	1.3 ± 0.3	19.5 ± 1.7
1.0	14.1 ± 1.4	30.3 ± 2.9
2.0	19.3 ± 2.3	85.6 ± 4.3

Figure 10 - TABLE 2

METHODS FOR IDENTIFYING FRAGILE HISTIDINE TRIAD (FHIT) INTERACTION AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS AND STATEMENT REGARDING SPONSORED RESEARCH

The present invention claims the benefit of PCT application No. PCT/US/2008/081294 filed Oct. 27, 2008 which claims priority to the provisional patent application Ser. No. 60/000,480 filed Oct. 26, 2007.

This invention was made with government support under NCI Grant Nos. CA77738 and CA78890. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The FHIT gene encompasses the most active common fragile site at chromosome 3p14.2 (1, 2). Fhit expression is lost or reduced in a large fraction of most types of human tumors due to allelic loss, genomic rearrangement, promoter hypermethylation, or combinations thereof (3, 4). Fhit knockout mice show increased susceptibility to cancer development (5, 6) and FHIT gene therapy prevents tumors in carcinogen-exposed Fhit-deficient mice (7, 8). Fhit restoration by stable transfection in cancer cells has little effect in vitro, unless cells are exposed to stress, including the stress of the nude mouse environment in vivo (9); viral-mediated Fhit restoration, a process that simultaneously supplies stress and Fhit expression, suppresses tumorigenesis in vivo and triggers apoptosis of many types of malignant cells in vitro (10-13), including lung cancer cells.

In lung hyperplastic lesions, DNA damage checkpoint genes are already activated, leading to selection for mutations in checkpoint proteins and neoplastic progression (14, 15). Evidence of DNA alteration at FRA3B within FHIT accompanied the hyperplasia and checkpoint activation. Loss of FHIT alleles occurs in normal appearing bronchial epithelial cells of smokers, prior to pathologic changes or alterations in expression of other suppressor genes (16-18).

Fhit expression is down-regulated by exposure to DNA damaging agents (19) and Fhit plays a role in response to such agents (20, 21), with Fhit-deficient cells escaping apoptosis and accumulating mutations.

Although Fhit expression triggers apoptosis in several experimental models through caspase-dependent mechanisms involving extrinsic and intrinsic apoptotic pathways, little is known about early events in this process and how Fhit loss is involved in tumor initiation.

Therefore, there is a need for methods for altering the expression of FHIT in subjects in need thereof. There is also a need for compositions that are useful to alter the expression of FHIT in subjects in need thereof.

SUMMARY OF THE INVENTION

In a broad aspect, there is provided methods which identify proteins that interact directly with Fhit to effect downstream signal pathways culminating in apoptosis. In one embodiment, proteins within cells were chemically cross-linked after infection of lung cancer cells with AdFHIT-His6 virus. The proteins linked to Fhit and pathways affected by them were identified and characterized.

In another broad aspect, there is provided herein a method of diagnosing whether a subject has, or is at risk for developing, a cancer associated disorder, comprising measuring the level of at least fragile histidine triad (Fhit) gene in a test sample from the subject, wherein an alteration in the level of the Fhit gene product in the test sample, relative to the level of a corresponding Fhit gene product in a control sample, is indicative of the subject either having, or being at risk for developing, a cancer associated disorder.

Various objects and advantages of this invention will become apparent to those skilled in the art from the following detailed description of the preferred embodiment, when read in light of the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

FIGS. **1**A-**1**H—Subcellular localization of Fhit protein in cytosol and mitochondria.

FIG. **1**A, immunofluorescence microscopy was performed with anti-Fhit serum on H1299 cells (D1) treated with PonA for 48 h; Fhit staining was detected using fluorescein isothiocyanate (green)-conjugated anti-rabbit immunoglobulin (IgG); Mito-Tracker Red staining, which identifies mitochondria, shows partial colocalization with Fhit. The yellow color on the fourth panel (lower right) shows the co-localizations points.

FIG. **1**B, immunoelectron microscopy of A549 AdFHIT (left) or AdFHIT-His_6-infected cells (right) performed with a penta-His antibody shows Fhit mitochondrial localization (right); A549 cells infected with AdFHIT served as control and show only a few scattered grains (left panel).

FIG. **1**C, immunoblot analysis of AdFHIT-infected A549 subcellular fractions using anti-Fhit indicates Fhit protein distribution in the cytosol, membranes, cytoskeleton, and mitochondria.

FIG. **1**D, immunoblot analysis of proteins from mitochondria of A549 cells infected with AdFHIT-His_6 after treatment with sodium carbonate (FIG. **1**E) and increasing concentrations of digitonin (FIG. **1**F) indicates that Fhit is mainly distributed in mitochondrial matrix; filters were probed with Fhit and CoxIV antisera; lanes in FIG. **1**F represent supernatants after treatment with 0, 0.10, 0.15, and 0.20% digitonin.

FIG. **1**G, immunoblot analyses of subcellular fractions from MKN74/E4 and MKN74/A116 cells (stably expressing exogenous Fhit), and FIG. **1**H, HCT116 (an endogenous Fhit-positive colon cancer cell line) using anti-Fhit, confirms Fhit mitochondrial localization; GAPDH and CoxIV antisera served as controls.

FIGS. **2**A-F—Exogenous and endogenous Fhit forms a complex with endogenous Hsp60, Hsp10, and Fdxr proteins. Protein complexes, isolated with recombinant Fhit-His_6 protein, were separated on polyacrylamide gels and probed with antisera against Hsp60 (FIG. **2**A), Hsp10 (FIG. **2**B), and Fdxr (FIG. **2**C); in the latter panel, prepared after mitochondria isolation, it is shown that Fhit recruits Fdxr in the mitochondria in a time-dependent manner. Filters were loaded with protein isolated after infection of A549 cells with AdFHIT-His_6 with or without DSP.

FIG. **2**D, coimmunoprecipitation with anti-Hsp60 after infection of A549 cells with AdFHIT; filters were probed with Hsp60, Fhit, and Hsp10 antisera.

FIG. **2**E, A549 cells were co-transfected with the VS-tagged FDXR gene and FHIT plasmids; immunoprecipitation was with anti-V5 and detection with Fdxr and Fhit antisera.

FIG. **2**F, immunoprecipitation and immunoblot detection of endogenous interactor proteins (Fdxr and Hsp10) from DSP-treated Fhit-positive HCT116 cells. Filters were probed with antisera against each target protein. Endogenous Fhit co-precipitated with Hsp10 and Fdxr.

FIGS. **3**A-D—Knockdown of Hsp60/Hsp10 reduces the level of Fhit in the mitochondria.

FIG. **3**A, nickel-H6 pull down experiment of A549 cells AdFHIT-His_6 infected on subcellular fractions (Cytosol and Mitochondria) using H6 antibody; lysates were incubated

with nickel beads to isolate the DSP cross-linked Fhit-His_6 protein complex and loaded on a 4-20% polyacrylamide gel. 24 h after infection, the Hsp60-Fhit complex was present in both compartments; 48 h after infection, the complex was detectable again in both compartments and the increase of Fhit complex proteins appears related to the increase of Fhit protein at 48 h after AdFHIT-His_6 infection, with a slight increase in the mitochondria (densitometry analysis on input samples was performed).

FIG. **3**B, immunoblot analysis of Hsp60, Hsp10, Fhit, and GAPDH in Fhit-positive D1 cells after 72 h of Hsp60/Hsp10 silencing showing Fhit, Hsp60, and Hsp10 levels after a CHX chase (30 μg/ml) for 1-12 h.

FIG. **3**C, immunoblot analysis of cytosol/mitochondrial protein fractions of A549 cells 72 h after transfection with Hsp60 and Hsp10 siRNAs and 24 h after AdFHIT infection at m.o.i. 1, with Hsp60, Hsp10, Fhit, GAPDH, and CoxIV antisera. Hsp60/10 silencing does not appear to affect the Fhit cytosolic level, but is associated with a decrease of Fhit in the mitochondrial fraction. Scrambled (Scr) siRNAs were used as controls.

FIG. **3**D, subcellular fractionation and immunoprecipitation of "endogenous" Fhit complex proteins. PonA-induced D1 and E1 cells, with and without peroxide treatment, were fractionated into cytosol and mitochondria and subcellular fractions assessed for the presence of Fhit and interactors (left side) at 48 h after induction; 25 μg of proteins were loaded per lane. Endogenous Hsp60 co-precipitated Fhit and Fdxr.

FIGS. **4**A-F—Fhit expression induces intracellular ROS generation after treatment of cells with peroxide.

FIG. **4**A, fluorescence-activated cell sorter (FACS) analysis for ROS assessment in A549 cells 48 h after transfection with FHIT plasmid, with and without a 5-h H_2O_2 treatment. Empty vector-transfected cells served as control. Intracellular superoxide was determined according to the fluorescence of ethidium as a result of oxidation of hydroethidine by O_2. M2 refers to the fraction of ROS positive cells.

FIG. **4**B, FACS analysis for ROS assessment by the fluorescence produced from the oxidation of hydroethidine in D1 and E1 cells; 48 h after PonA treatment, cells were treated for 5 h with 0.5 and 1.0 mM H_2O_2 and oxidative stress was measured; % positive refers to the fraction of fluorescent cells, indicating ROS. These experiments were repeated three times with similar results.

FIG. **4**C, increased green fluorescent DCF signal in H1299 Fhit-expressing cells (D1) under stress conditions. Cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate, a ROS indicator that can be oxidized in the presence of ROS to the highly green fluorescent dye DCF, at 48 h after Fhit induction and after a 5-h H_2O_2 treatment of E1 and D1 cells (magnification ×40).

FIG. **4**D, MTS cell viability assays were performed on E1 and D1 cells. Cells were treated with PonA for 48 h and then with increasing concentrations of H_2O_2 (0.125, 0.25, and 0.5 mM) for 4 h. Analysis was at 24 h after H_2O_2 treatment. Columns report the average of four experiments ±S.E. Each point was measured in quadruplicate and standard deviation calculated; $p<0.05$ was considered significant.

FIG. **4**E, FACS analysis of D1 and E1 cell cycle kinetics at 48 h after oxidative stress treatment. Cells were treated with PonA for 48 h and then with increasing concentrations of H_2O_2 (0.25 and 0.5 mM) for 4 h. Analysis was at 48 h after H_2O_2 treatment. All experiments were performed twice in triplicate.

FIG. **4**F, colony formation assay of H1299/D1 and H1299/E1 cells after 5 mM PonA stimulation and a 5-h H_2O_2 treatment at the indicated concentrations.

FIGS. **5**A-H.—Apoptosis triggered by Fhit viral transduction can be mediated by its interaction with Fdxr.

FIG. **5**A, immunoblot analysis with antisera against Fdxr, Fhit, and GAPDH. Proteins were extracted from E1 (control) and D1 cells 48 h after treatment with PonA.

FIG. **5**B, immunoblot analysis of Fdxr expression in D1 and E1 cells after a 4-h treatment with 25 μA MG132, a proteasome inhibitor. GAPDH detection shows equal protein loading.

FIG. **5**C, immunoblot analysis of Fdxr, Fhit, and GAPDH in D1, expressing Fhit, and E1 cells, showing Fdxr level after CHX chase (30 μg/ml) for 4-12 h. Densitometry based on GAPDH levels shows enhanced stability of Fdxr in the presence of Fhit.

FIG. **5**D, FACS analysis of $FDXR^{+/+/+}$ and $FDXR^{+/-/-}$ cell cycle kinetics after infection with AdFHIT m.o.i. 50 and 100. The experiment was performed 48 h after infection and was repeated three times with similar results. Profiles of AdGFP-infected cells were similar to those of non-infected cells (not shown).

FIG. **5**E, immunoblot analysis showing expression of Fdxr, Fhit, and GAPDH after infection of $FDXR^{+/+/+}$ and $FDXR^{+/-/-}$ with AdFHIT m.o.i. 50 and 100. Proteins were extracted 48 h after infection.

FIG. **5**F, real-time RT-PCR analysis for FDXR expression at 48 h after AdFHIT m.o.i. 50. The PCR product was normalized to GADPH and Actin expression and each point was repeated in quadruplicate; differences between control and Fhit positive samples were not significant.

FIG. **5**G, caspase 3 and Parp1 activation. Immunoblot analysis, using Fhit, caspase 3, Parp1 antisera, of total cell lysates from HCT116 FDXR cells 48, 72, and 96 h after infection with AdFHIT and AdGFP at m.o.i. 50. GAPDH and CoxIV served as internal protein markers.

FIG. **5**H, immunoblot analysis, using Fhit and cytochrome c antisera, of cytosol/mitochondria fractions from HCT116 FDXR cells 48, 72, and 96 h after infection with AdFHIT and AdGFP at m.o.i. 50. GAPDH and β-actin served as internal protein markers.

FIGS. **6**A-E—Fhit enhances the sensitivity of cancer cells to paclitaxel and cisplatin.

MTS assays performed on E1 and D1 cells. Cells were treated with PonA for 48 h and then treated with paclitaxel (50-500 ng/ml) (FIG. **6**A) or cisplatin (0.05-0.2 mM) (FIG. **6**B) for 24 or 48 h. Bars report the average of four experiments ±S.E. Each point was measured in quadruplicate and standard deviation calculated; asterisks next to brackets in FIG. **6**A and FIG. **6**B indicate statistically significant differences in drug response of D1 and E1 cells, $p<0.05$.

FIG. **6**C and FIG. **6**D, the graphs show representative results of flow cytometry analyses of E1 and D1 cells. Cells were treated with PonA for 48 h and then with paclitaxel (50-500 ng/ml) (FIG. **6**C) or cisplatin (0.05-0.2 mM) (FIG. **6**D). Each data point was measured in triplicate at 24, 48, and 72 h (data shown for 48 h).

FIG. **6**E, caspase 3 and Parp1 cleavage: immunoblot analyses, using Fhit, caspase 3, and Parp1 antisera, of total cell lysates from PonA-induced D1 cells after 48 h of treatment with paclitaxel (50 and 100 ng/ml) or cisplatin (0.05 and 0.1 mM). GAPDH served as loading control.

FIG. **7**. TABLE 1 Candidate Fhit protein partners isolated through mass spectrometry. Proteins selectively captured in the A549 AdFHIT-H_6-infected cells sample. Amino acid sequence of identified peptides, Mascot scores, and protein sequence coverage are listed.

FIGS. **8**A-**8**C. Ad-His6 biological activity is comparable to AdFHIT.

FIG. **8**A, Western blot analysis of A549 cells infected with Ad-His6, MOI 20. Fhit-His protein was detected by antipentaHis and antiFhit serum. Both Ad FHIT and Ad-His6 carry a GFP cDNA regulated by a CMV5 promoter through an internal ribosome entry sequence downstream of FHIT. γ-tubulin was used to normalize sample loading.

FIG. **8**B, Flow cytometry analysis of A549 cells 96 hr after infection with Ad-His6, MOI 15. Upper panel indicates the subG1 DNA content of infected cells (experiment repeated thrice; average values of subG1 fractions 22%+/−4.3 for Ad

FHIT, 29%+/−5 for Ad-His6; the difference is not statistically significant; lower panel shows percentages of cells with mature caspase-3, an indication of apoptosis. The extent of cell death in A549 cells infected with Ad-His6 is comparable to the result obtained after infection with Ad FHIT.

FIG. **8**C, In vivo cross-linking of Fhit-His6. Silver staining of gel with cell lysates after His6 pull down and cross-link reversal conditions, separated by 4-20% gradient SDS-PAGE. Internal negative controls included His6 pull down of Ad FHIT infected cells (cross-linked, CL) and Ad-His6 infected cells (not cross-linked, NT).

FIGS. **9**A-**9**F. Initial validation of candidate Fhit protein partners identified through nanobore LC-MS/MS. Selected ion chromatograms (SIC) for AdFHIT-His6 and control samples are shown. The six SICs pairs reportion currents of the six following m/z values: 1) 672.8 (peak at retention time 30 min. was identified as tryptic peptide TVIIEQSWGSPK [SEQ ID NO: 5] belonging to Hsp60), 2) 685.4 (peak at retention time 32 min. identified as tryptic peptide LGPALATGNVVVMK [SEQ ID NO: 22] belonging to Aldh2), 3) 617.3 (peak at retention time 39 min. identified as tryptic peptide IFGVTTLDIVR [SEQ ID NO: 10] belonging to Mdh), 4) 658.4 (peak at retention time 26 min. identified as tryptic peptide VLQATVVAVGSGSK [SEQ ID NO: 19] belonging to Hsp10), 5) 551.7 (peak at retention time 28 min. identified as tryptic peptide EIDGGLETLR [SEQ ID NO: 15] belonging to Etfb), 6) 598.3 (peak at retention time 23 min. identified as tryptic peptide FGVAPDHPEVK [SEQ ID NO: 23] belonging to Fdxr). Peptides of interest, indicated by red arrows, are exclusively present in Ad-His6 sample.

FIG. **10**. TABLE 2, Fhit induces generation of ROS in MKN74 gastric cancer cells. ROS assessment was performed with MKN74A116, a human gastric cancer cell line carrying a p53 mutant allele and expressing exogenous Fhit; Fhit-negative MKN74E4 cells were used as a control. To induce ROS generation, we treated MKN74 cells for 5 hr with 0.5, 1.0 and 2.0 mM H_2O_2. Results indicate a significantly higher rate of ROS generation in cells expressing exogenous Fhit compared to controls; toxicity was observed in Fhit-expressing cells after 2 mM H_2O_2 treatment. Numbers report the average of four experiments ±S.E.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not intended to limit the scope of the current teachings. In this application, the use of the singular includes the plural unless specifically stated otherwise.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Also, the use of "comprise", "contain", and "include", or modifications of those root words, for example but not limited to, "comprises", "contained", and "including", are not intended to be limiting. The term "and/or" means that the terms before and after can be taken together or separately. For illustration purposes, but not as a limitation, "X and/or Y" can mean "X" or "Y" or "X and Y".

The term "combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, ACB, CBA, BCA, BAC, or CAB.

As used herein interchangeably, "gene product," "DNA" and "gene," are used herein interchangeably.

The following abbreviations may be used herein: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DSP, dithiobis (succinimidyl propionate); LC-MS/MS, liquid-chromatography tandem mass spectrometry; Fdxr, ferredoxin reductase; PonA, ponasterone A; m.o.i., multiplicity of infection; ROS, reactive oxygen species; FU, 5-fluorouracil; DCFH-DA, dichlorofluorescein-diacetate; DCF, 2',7'-dichlorofluorescein; CHX, cycloheximide; siRNA, small interfering RNA; RT, reverse transcriptase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way. All literature and similar materials cited in this application, including patents, patent applications, articles, books, treatises, and internet web pages are expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated literature and similar materials defines or uses a term in such a way that it contradicts that term's definition in this application, this application controls.

Fhit protein is lost in most cancers, its restoration suppresses tumorigenicity, and virus-mediated FHIT gene therapy induces apoptosis and suppresses tumors in preclinical models. Protein cross-linking and proteomics methods are used to characterize a Fhit protein complex involved in triggering Fhit-mediated apoptosis. The complex includes Hsp60 and Hsp10 that mediate Fhit stability and may affect import into mitochondria, where it interacts with ferredoxin reductase, responsible for transferring electrons from NADPH to cytochrome P450 via ferredoxin. Viral-mediated Fhit restoration increases production of intracellular reactive oxygen species, followed by increased apoptosis of lung cancer cells under oxidative stress conditions; conversely, Fhit-negative cells escape apoptosis, carrying serious oxidative DNA damage that may contribute to an increased mutation rate. Characterization of Fhit interacting proteins has identified direct effectors of the Fhit-mediated apoptotic pathway that is lost in most cancers through loss of Fhit.

Earlier searches for Fhit-interacting proteins pointed to several candidate proteins, none of which we could confirm as interactors by co-immunoprecipitation experiments, including Ubc9, α-tubulin, and Mdm2 (35-37). To readdress the question of Fhit protein interactors, the following was used: adenovirus-transduced Fhit-His_6 for Fhit complex purification after cross-linking, and Fhit-linked proteins, Hsp60, Hsp10, and Fdxr, were identified; subcellular location of these proteins suggested that mitochondria might be foci of Fhit activity. Hsp "stress proteins" as molecular chaperones perform functions such as protein translocation, folding, and assembly (38). The finding that Fhit interacts with Hsp60/Hsp10 after AdFHIT infection suggests that the Hsp complex may be important for Fhit stability, and possibly for its correct folding to import it into mitochondria, prior to activation of the apoptotic pathway, a suggestion we investigated by knocking down expression of Hsp60, Hsp10, or both in AdFHIT-infected lung cancer cells; Fhit stability was assessed after CHX chase in H1299 D1 cells, the lung cancer cell line expressing inducible Fhit. The level of Fhit protein in isolated mitochondria after knockdown of both Hsp60 and -10 was reduced, strengthening the proposal that Fhit-Hsp60/10 interaction is involved in Fhit stabilization and/or in correct folding for importation into mitochondria.

Targeted disruption of the FDXR gene in HCT116 colon cancer cells showed that it was essential for viability; reduction of the gene copy number resulted in decreased sensitivity to 5-fluorouracil-induced apoptosis (29) and FDXR is a target gene of the p53 family (30). Overexpression of Fdxr-sensitized colon cancer cells to H_2O_2, 5-fluorouracil, and doxorubicin-induced cell death, indicating that Fdxr contributes to p53-mediated apoptosis through generation of oxidative stress in mitochondria. Thus, activated p53 induces apoptosis

in response to cellular stresses in part through ROS, and simultaneously p53 increases transcription of the FDXR gene, which in turn enhances p53 function by increasing ROS-induced apoptosis (29, 30).

Now shown herein is the presence of Fhit in the mitochondrial fraction; when Fhit is overexpressed or Fhit-expressing cells are stressed, Fhit can protect Fdxr from proteosomal degradation, leading to an increase in the Fdxr protein level, which is associated with generation of ROS and followed by apoptosis. Fhit does not affect the FDXR transcriptional level but may affect stability of the protein. In H1299 cells, missing both Fhit and p53, Fdxr overexpression increases sensitivity to ROS-induced cell death, and H1299 cells expressing inducible Fhit or p53 are sensitive to ROS-induced cell death; cancer cells missing Fhit, p53, or both would lack ways to increase Fdxr expression, and would be less sensitive to oxidative damage and would survive.

Discovery of the mitochondrial function of Fhit in apoptosis through interaction with Fdxr now extends functional parallels of the important tumor suppressors, Fhit and p53, lost sequentially in most cancers and involved in response to DNA damage, and illuminates their differences, with p53 acting as a transcriptional and Fhit a post-transcriptional Fdxr regulator. Delineation of direct downstream effectors of the Fhit suppressor pathway will lead to mechanistic studies of Fhit function that may influence preventive and therapeutic strategies to activate the Fhit pathway.

The finding that ROS generation is crucial for Fhit-mediated apoptosis emphasizes the importance of Fhit loss as a negative prognostic factor in various clinical settings; for example, assessment of Fhit status in preneoplastic or neoplastic conditions may be predictive of responses to antioxidant treatments.

To identify proteins that interact with Fhit to effect downstream apoptotic pathways, the inventors herein cross-linked proteins within cells after viral-mediated Fhit overexpression in lung cancer cells, and characterized proteins associated with Fhit and the pathways affected by them.

Results.

Isolation of a Fhit Protein Complex—To identify Fhit-interacting proteins, we generated an adenovirus carrying FHIT cDNA modified at its 3' end with a sequence encoding a His_6 epitope tag (AdFHIT-His_6). The biological activity of this tagged Fhit protein expressed in A549 cells was comparable with wild-type Fhit activity (FIG. **8**).

A549 lung cancer-derived cells, which are susceptible to Fhit-induced apoptosis (10), were infected with AdFHIT or AdFHIT-His_6 and treated with DSP, a cross-linker that crosses membranes and fixes proteins in complex in vivo. Cells were lysed and proteins isolated with nickel beads avid for the His_6 epitope tag. Purified proteins were treated with dithiothreitol to cleave DSP and dissociate the complex, and digested by trypsin; protein constituents were identified by LC-MS/MS (FIG. **7**—Table 1 and FIG. **9**).

TABLE 1

Protein	Accession no.	Molecular mass kDa	Function/ category	Subcellular localization	No. identified peptides	Peptide sequences	SEQ ID NO:	Protein Mascot score	Sequence coverage
Hsp60	NP_002147	60	60-kDa Heat shock protein	Cytosol/ mitochondria	6	VGEVIVTK	1	239	10%
						LSDGVAVLK	2		
						IGIEIIKR	3		
						VTDALNATR	4		
						TVIIEQSWGSPK	5		
						VGGTSDVEVNEKK	6		
Malate dehydrogenase (Mdh)	NP_005909	33	Catalyzes the reversible oxidation of malate to oxaloacetate	Mitochondrial matrix	8	ANTFVAELK	7	193	28%
						IQEAGTEVVK	8		
						VNVPVIGGHAGK	9		
						IFGVTTLDIVR	10		
						FVFSLVDAMNGK	11		
						GCDVVVIPAGVPR	12		
						AGAGSATLSMAYAGAR	13		
						GYLGPEQLPDCLK	14		

TABLE 1-continued

Protein	Accession no.	Molecular mass kDa	Function/ category	Subcellular localization	No. identified peptides	Peptide sequences	SEQ ID NO:	Protein Mascot score	Sequence coverage
Electron transfer flavor protein (Etfb)	NP_001976	28	Specific electron acceptor for mitochondrial dehydro-genases	Mitochondrial matrix	3	EIDGGLETLR	15	96	12%
						VETTEDLVAK	16		
						LSVISVEDPPQR	17		
Hsp10	AAC 96332	10	10-kDa Heat shock protein	Cytosol/ mitochondria	3	GGEIQPVSVK	18	92	34%
						VLQATVVAVGSGSK	19		
						VVLDDKDYFLFR	20		
Mitochon-drial aldehyde dehydro-genase 2 (Adh2)	NP_000681	55	Second enzyme of the major oxidative pathway of alcohol metabolism	Mitochondrial matrix	2	LADLIER	21	75	4%
						LGPALATGNVVVMK	22		
Ferredoxin reductase (Fdxr)	P22570	54	First electron transfer protein in all the mitochondrial p450 systems	Mitochondrial matrix	1	FGVAPDHPEVK	23	47	2%

Six proteins were identified, all with mitochondrial localization: Hsp60 and 10, ferredoxin reductase (Fdxr), malate dehydrogenase, electron-transfer flavoprotein, and mitochondrial aldehyde dehydrogenase 2; Hsp60 and Hsp10 are also distributed in the cytosol (23).

Fhit Subcellular Localization—Because candidate Fhit interactors are mitochondrial proteins, the inventors herein determined if Fhit, which lacks a mitochondrial localization signal, localized in these organelles. Fhit negative H1299 lung cancer cells carrying an inducible FHIT cDNA (D1 cells) were treated with the inducer, PonA for 48 h and indirect immunofluorescence detection of Fhit subcellular location was assessed using anti-Fhit serum and MitoTracker Red 580, a marker of mitochondria; Fhit fluorescent signal (green staining, FIG. **1**A) was cytoplasmic and partly co-localized (yellow staining, FIG. **1**A, lower right) with MitoTracker Red dye, indicating that exogenous Fhit localized to mitochondria and cytosol. Anti-Fhit specificity was confirmed by absence of green fluorescence in the Fhit negative H1299 clone E1 cells (not shown). To confirm mitochondrial localization, A549 cells infected with AdFHIT-His_6 or AdFHIT at m.o.i. 20 were examined by immunoelectron microscopy 48 h later, by anti-pentaHis staining; FhitHis6-transduced cells demonstrated significant numbers of gold particles in mitochondria (FIG. **1**B, right panel), whereas AdFHIT-transduced cells showed sparse reactivity (FIG. **1**B, left panel).

To assess Fhit submitochondrial localization, mitochondria were purified from A549 cells infected with AdFHIT m.o.i. 1, as described above. The sodium carbonate procedure is a nondestructive approach that allows effective release in the supernatant of both soluble proteins and peripheral membrane proteins from intracellular membranes after inducing the generation of open sheets of membranes; furthermore, it allows recovery of integral proteins with the membranes (pellet) (24).

FIG. **1**E shows that Fhit was only detectable in the soluble fraction. To further define Fhit submitochondrial localization, mitochondria were treated with 0.10 and 0.15% digitonin to selectively disrupt mitochondrial outer membrane, releasing proteins contained in the intermembrane space and the matrix; as shown in FIG. **1**F, gradual disruption of outer and inner membranes releases increasing amounts of Fhit protein, suggesting that Fhit is mainly distributed either at the luminal side of the inner membrane or in the matrix of mitochondria. Mitochondrial localization was confirmed in gastric cancer-derived MKN74A116 cells stably expressing exogenous Fhit (9) and in HCT116 colon cancer cells expressing endogenous Fhit (FIG. **1**G and FIG. **1**H).

Fhit Interacts with Hsp60, Hsp10, and Fdxr—Among candidate interactor proteins, the inventors focused on Hsp60 and Hsp10 as possible chaperonins and on Fdxr, a mitochondrial respiratory chain protein transactivated by p53 and involved in responses to therapeutic drugs (25). To validate interactions, A549 cells were infected with AdFHIT or AdFHIT-His6 at m.o.i. 20, with or without DSP. Fhit complexes were purified through the His_6 tag and co-purified proteins were detected with antisera against Hsp60, Hsp10, and Fdxr; Hsp60 and Fdxr were detected only in lysates of cells exposed to DSP (FIG. **2**A and FIG. **2**C), whereas Hsp10 was also detectable without cross-linking (FIG. **2**B).

A time course experiment after infection showed recruitment of Fdxr by Fhit (FIG. **2**C); also, endogenous Hsp60 co-immunoprecipitated Fhit and Hsp10 in the absence of DSP (FIG. **2**D).

To verify specificity of interactions we generated an FDXR cDNA expression plasmid with a 3'V5 epitope tag. A549 cells were co-transfected with FDXR-V5 and FHIT plasmids, and proteins were precipitated with monoclonal anti-V5; co-precipitated Fhit was detectable only after DSP cross-linking (FIG. **2**E).

To determine whether these proteins also interact with endogenous Fhit, the inventors immunoprecipitated each endogenous candidate interactor protein from DSP-treated Fhit-positive HCT116 cells and looked for co-precipitation of endogenous Fhit (FIG. **2**F).

Endogenous Fhit co-precipitated with Hsp10 and Fdxr, confirming the presence of endogenous Fhit in mitochondria and its interaction with endogenous chaperones and respiratory chain protein in the absence of stress.

Hsp60/10 Interaction Affects Fhit Stability and/or Mitochondrial Import—Hsp60 and -10 are molecular chaperones found in complex (26) and may be important for folding and import of proteins into mitochondria. The inventors herein now believe that the Hsp60/10 complex was responsible for Fhit correct folding and mitochondrial addressing.

To investigate the location of these interactions, A549 cells were infected with AdFHIT-His_6 m.o.i. 5 and protein lysates were collected from cytosol and mitochondrial fractions after cross-linking. Complexes were isolated by Fhit-H6-nickel pull down, separated on a polyacrylamide gel, and filters probed with Hsp60 and Fhit antisera. At 24 and 48 h after infection interaction with Hsp60 is observed in the cytosol and mitochondria (FIG. **3**A) commensurate with the increase in Fhit expression at these times (Input), as shown in FIG. **3**A.

To determine whether the Fhit-Hsp60/10 interaction is important for the stability of the Fhit protein, H1299 with inducible Fhit expression (D1 cells) were transfected with Hsp60 and Hsp10 siRNAs and 72 h after transfection a CHX chase was performed at 1, 6, and 12 h and Fhit protein expression was assessed and compared with cells transfected with the scrambled sequence.

As shown in FIG. **3**B at 6 and 12 h of CHX after Hsp60 and Hsp10 silencing, there is a strong reduction of Fhit expression (from 1 to 0.4 at 1 and 12 h, respectively). Next, Hsp60 and 10 siRNAs were transfected into A549 cells individually or in combination; 24 h later, cells were infected with AdFHIT m.o.i. 1 and cytosol and mitochondria were fractionated 24 h later. After silencing both Hsps, the Fhit level was unaffected in the cytosol but reduced in mitochondria compared with control (FIG. **3**B), showing that the Hsp60/10 complex may mediate virally transduced Fhit stabilization and mitochondrial localization. It is also true that if Hsp60 and Hsp10 are involved in Fhit stability after Fhit viral transduction, the cellular compartment with less Fhit would be affected by a decrease in Fhit stability. The inventors herein also examined the Fhit complex in H1299 D1 cells expressing inducible Fhit, with Fhit negative E1 cells as control; 48 h after Fhit induction in D1 cells (FIG. **3**C, left panel), distribution of the Fhit complex proteins was similar in the cytosol and mitochondria of D1 and E1 cells, with and without H_2O_2.

Hsp60 was immunoprecipitated from total cell lysates of these cells at 48 h after PonA induction, with or without H_2O_2, and coprecipitated Fhit and Fdxr (FIG. **3**C, right panel). Induction of Fhit expression in D1 cells does not cause biological changes in vitro; thus the Fhit complex does not form as a consequence of apoptosis. A time course experiment was performed in D1 cells after PonA-induced Fhit expression, with and without stress conditions, to determine whether there were biological changes in Fhit protein interactors. The co-inventors did not detect changes in localization after Fhit expression.

Fhit Induces Generation of Reactive Oxygen Species (ROS)—Fdxr, a 54-kDa flavoprotein, is located on the matrix side of the inner mitochondrial membrane, and is responsible for transferring electrons from NADPH, via the single electron shuttle ferredoxin-cytochrome P450, to substrates (27). Under substrate-limiting conditions, electrons leak from this shuttling system and generate ROS (28). Fdxr mediates p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells, through generation of ROS (29, 30), potent intracellular oxidants, and regulators of apoptosis (31).

The inventors herein then investigated determine whether ROS production could be involved in Fhit-mediated apoptosis. Overexpression of Fdxr increases sensitivity of tumor cells to apoptosis on H_2O_2 treatment, through ROS production (29, 30). The inventors examined ROS production in A549 cells, with and without H_2O_2 treatment, after transient transfection with the FHIT expression plasmid. Intracellular superoxide was assessed by measuring ethidium fluorescence, as a result of oxidation of hydroethydine by superoxide. Intracellular superoxide was measured 5 h after stimulation with increasing concentrations of H_2O_2. ROS generation was ~3 times higher (16.7 versus 5.4% at 0.5 mM H_2O_2 and 18.8 versus 7.7% at 1.0 mM H_2O_2) in FHIT-transfected cells. 2 mM H_2O_2 was toxic to Fhit-expressing but not to non-expressing cells (FIG. **4**A).

A similar experiment was performed with p53 and Fhit negative lung cancer-derived H1299 D1 and E1 clones carrying PonA-inducible FHIT and empty vector expression plasmids, respectively; the cells were treated with 5 μM PonA and at 48 h treated with 0.5 and 1.0 mM H_2O_2; the % ROS-positive cells was higher in Fhit-positive D1 cells than in E1 control cells (20 versus 3.5% at 0.5 mM H_2O_2, and 78 versus 25% at 1.0 mM H_2O_2, respectively) (FIG. **4**B).

These results were paralleled by experiments with human gastric cancer-derived cells, MKN74A116 (FIG. **10**), which express mutant p53 (32) and stably express exogenous Fhit (9).

To further study the generation of ROS after Fhit reconstitution during oxidative stress, DCFH-DA was used to measure the redox state of Fhit-overexpres sing cells. Peroxidases, cytochrome c, and Fe^{2+} can oxidize DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of H_2O_2; thus, DCF indicates H_2O_2 levels and peroxidase activity. Increased DCF fluorescence was detected in D1 cells compared with E1 cells under stress conditions (FIG. **4**C).

The decreased cell viability after H_2O_2 treatment in Fhit-expressing cells was also assessed by an MTS cytotoxicity assay 24 h after H_2O_2 treatment. H_2O_2 treatment caused reduced cell viability or growth arrest in both E1 and D1 cells, but this phenotype was more pronounced in D1 cells (FIG. **4**D).

To determine whether H_2O_2 treatment with or without Fhit could affect cell viability or cell cycle kinetics we performed flow cytometry (FIG. **4**E); when Fhit was present under stress conditions there was a consistent increase of G_2/M arrest at 48 h after 0.25 and 0.5 mM H_2O_2 treatment, 45.5 and 49.5%, respectively, compared with 27.5 and 29% of E1 cells under the same conditions.

To assess if the G_2/M arrest could affect long-term viability of the cells, a colony assay was performed (FIG. **4**F). No colonies were detected in Fhit-express sing cells after exposure to 0.25 mM or higher concentrations of H_2O_2.

Fhit-induced ROS Generation Is Fdxr-dependent—To evaluate the role of Fdxr in Fhit-mediated ROS generation, the inventors examined the Fdxr level in D1 cells after Fhit induction and observed a 2.4-fold increase of its expression compared with E1 cells (FIG. **5**A), an increase that was not due to increased transcription as determined by real time RT-PCR (FIG. **5**F).

The inventors next measured the Fdxr level, with or without Fhit expression, in the presence of MG132, an inhibitor of proteasome degradation; 4 h after MG132 treatment a significant increase of Fdxr protein was observed in D1 cells compared with E1 cells (FIG. **5**B), showing that Fhit protects Fdxr from proteasome degradation.

The rate of Fdxr degradation in the presence or absence of Fhit protein was evaluated by the 4-12-h CHX chase (FIG. **5**C); the rate of Fdxr degradation was higher in Fhit-negative E1 cells (declining from 1 to 0.3) compared with D1 cells, with no significant decrease. Thus, the inventors herein now believe that Fhit prevents destabilization of the Fdxr protein by protecting it from proteasome degradation.

HCT116 colon cancer cells, which express endogenous wild-type p53 and Fhit and carry three FDXR alleles ($FDXR^{+/+/+}$), and $HCT116FDXR^{+/-/-}$ cells with two alleles knocked-out (28), were used to determine whether AdFHIT-induced apoptosis is influenced by the Fdxr expression level; the FDXR null condition was not compatible with viability (29).

These cells were infected with AdFHIT m.o.i. 50 or 100 and assessed for apoptosis at 48 and 72 h post-infection. Wild-type HCT116 cells ($FDXR^{+/+/+}$) were susceptible to exogenous Fhit-mediated apoptosis in a dose-dependent manner, as the fraction of sub-G_1 cells was 12.1 and 18.8% at m.o.i. 50 and 100, respectively; $FDXR^{+/-/-}$ cells were refractory at 48 and 72 h (data not shown) to Fhit-induced cell death, with a sub-G_1 population of 4.7 and 4.3% at m.o.i. 50 and 100 (FIG. **5**D).

Fhit overexpression led to increased Fdxr protein levels in both $FDXR^{+/+/+}$ and $FDXR^{+/-/-}$ cells (FIG. **5**E) and $FDXR^{+/-/-}$ cells were committed to Fhit-mediated apoptosis by 72 h after infection.

The Fhit-mediated increase of Fdxr expression was not at the transcriptional level, as determined by real time RT-PCR (FIG. **5**F) and thus not related to the p53 transcriptional activation.

To better determine whether the sub-G_1 peak detected in $FDXR^{+/+/+}$ cells after AdFHIT infection was related to apoptosis induction, a time course experiment at 48, 72, and 96 h for caspase 3 and Parp 1 cleavage was performed and compared with AdGFP-infected cells (FIG. **5**G).

Caspase 3 cleavage and related Parp1 cleavage were observed at 48, 72, and 96 h after virus-mediated Fhit overexpression. The time course of cytochrome c release from mitochondria into cytosol was assessed after infection of HCT116 cells with AdFHIT m.o.i. 100 (FIG. **5**H); progressive cytochrome c release was observed in HCT116 FDXR cells compared with GFP-infected cells, indicating initiation of the apoptotic cascade in Fhit overexpressing HCT116 $FDXR^{+/+/+}$ cells.

Fhit Enhances ROS-related Effects of Chemotherapeutic Agents—Generation of intracellular ROS is an early event in the apoptosis of lung cancer cells induced by treatment with paclitaxel (33). The inventors tested paclitaxel on H1299 D1 and E1 cells with or without induced Fhit expression. After induction of Fhit expression, D1 cells were more sensitive to paclitaxel than E1 cells (FIG. **6**A) as measured by the MTS cell viability test. Cisplatin induces Fdxr expression and the cisplatin-induced apoptotic pathway is associated with ROS generation (34).

Fhit expressing D1 cells were more sensitive than E1 cells to cisplatin, measured by MTS assay at 24 and 48 h (FIG. **6**B).

To examine cell viability after drug treatment, we performed flow cytometry analysis (FIG. **6**, FIG. **6**C and FIG. **6**D); PonA-induced D1 and E1 cells treated with increasing paclitaxel concentrations (50-500 ng/ml) showed increasing sub-G_1 populations at 48 h: 9.6, 36, and 40%, respectively, for D1 cells compared with 4, 16.7, and 30% of E1 cells (FIG. **6**C).

Similarly, increasing cisplatin concentrations (0.05-0.2 mM) led to increased sub-G_1 populations at 48 h: 5, 16.2, and 30%, respectively, in D1 cells, compared with 2.3, 7, and 14.6% of E1 cells (FIG. **6**C).

At 24 and 72 h (data not shown) increased sub-G_1 populations in D1 compared with E1 cells were also detected. To determine whether the sub-G_1 fractions of D1 cells represented apoptotic cells, lysates were prepared from drug-treated cells at 48 h and immunoblot analysis performed for caspase 3 and Parp1 cleavage (FIG. **6**D).

Activated caspase 3 and related Parp1 cleavage was observed after paclitaxel (50 and 100 ng/ml) and cisplatin (0.05 and 0.1 mM) treatments compared with untreated cells (Ctrl). The inventors herein now believe that Fhit expression increases sensitivity to oxidative injury through participation with Fdxr in ROS generation.

Example I

Materials and Methods

Cells, Vectors, and Antisera—A549, H1299, MKN74-E4, and A116, and HCT116 cells were maintained in RPMI 1640 medium plus 10% fetal bovine serum and penicillin/streptomycin (Sigma). HEK293 cells (Microbix) used for preparation of recombinant adenoviruses were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and penicillin/streptomycin. AdFHIT-His6 virus was prepared as described in Example II herein. [His6—SEQ ID NO: 32] [penta-His—SEQ ID NO: 33].

Full-length FDXR was amplified from human brain cDNAs (Clontech), subcloned into the pcDNA3.1/V5-His-TOPO TA vector (Invitrogen) and sequenced; details are as described under supplemental Methods. Cells were transfected using Lipofectamine™ (Invitrogen) following the manufacturer's directions.

Western Blot Analysis—Immunoblot analyses were performed as described (13) using monoclonal anti-pentaHis (Qiagen); rabbit polyclonal anti-Fhit (Zymed Laboratories Inc.); rabbit polyclonal antisera against GFP, Hsp60, Hsp10, and cytochrome c (Santa Cruz Biotechnology); rabbit polyclonal anti-Fdxr (Abcam); monoclonal anti-CoxIV (Molecular Probes); anti-V5 (Sigma); anti-Parp1 (Santa Cruz Biotechnology); and anti-caspase 3 (Cell Signaling). Protein levels were normalized relative to β-actin or/and GAPDH[3] level, detected with appropriate antisera (Santa Cruz Biotechnology).

Mass Spectrometry Studies—Protein pellets were solubilized and digested by trypsin as described herein. Peptide mixtures were injected for LC-MS/MS analysis. After protein identification by data base search, inspection of LC-MS/MS data was undertaken to assess the exclusive presence of mass peaks belonging to candidate partner proteins in samples from cells infected with AdFHIT-His6.

Protein Interaction Analyses—Proteins were extracted in 15 mM Tris-Cl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM

EGTA, 0.1 mM dithiothreitol, 0.5% Triton X-100, 10 mg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride. Co-immunoprecipitation experiments, with or without dithiobis(succinimidyl propionate) (DSP), were performed by incubating 1 mg of total proteins with Hsp60, Hsp10, Fdxr, penta-His, and V5 antisera conjugated with Sepharose for 2 h at 4° C.; after washing, beads were boiled in 1×SDS sample buffer and proteins separated on 4-20% polyacrylamide gels (Bio-Rad), transferred to a poly(vinylidene difluoride) filter (Millipore), and probed with specific antisera.

Subcellular Localization of Fhit Protein—Fhit was sublocalized in ponasterone A (PonA)-induced, Fhit-expressing H1299 D1 cells by indirect immunofluorescence detection using anti-Fhit serum and by detection of FhitHis6 in A549 AdFHIT-His_6-infected cells in immunoelectron micrographs using anti-pentaHis. In fractionation studies, mitochondria were isolated with the Mitochondria/Cytosol Fractionation kit and the FractionPREP™ Cell Fractionation System was used to extract proteins from cytosol, membranes, nuclei, and cytoskeleton (Biovision Research Products). For submitochondrial localization according to the method of Dahéron et al. (22), mitochondria were resuspended in 0.1 M sodium carbonate, pH 11.5, on ice for 30 min with periodic vortexing and fractionated as described herein.

Flow Cytometry—HCT116 $FDXR^{+/+/+}$ and $FDXR^{+/-/-}$ cells were infected with AdFHIT or AdGFP at m.o.i. 50 and 100 and assessed at 48 h postinfection. PonA-induced H1299 D1 and E1 cells were treated with 0.25 and 0.5 mM H_2O_2 or with chemotherapeutic drugs and incubated for varying times, as indicated in the text and figures. For both experiments the cells were collected, washed with phosphate-buffered saline, and resuspended in cold 70% ethanol. For analysis, cells were spun down, washed in phosphate-buffered saline, and suspended in 0.1 mg/ml propidium iodide/Triton X-100 staining solution (0.1% Triton X-100, 0.2 mg/ml DNase-free RNase A) for 30 min at room temperature and analyzed by flow cytometry.

Assessment of Intracellular Reactive Oxygen Species (ROS)—Intracellular superoxide was measured through ethidium fluorescence as a result of oxidation by hydroethidine (dihydroethidium-HE; Molecular Probes). MNK74 stably Fhit expressing cells, A549 cells transiently expressing Fhit, and H1299 inducible Fhit expressing cells were treated with 0.5, 1.0, 2.0, and 4.0 mM H_2O_2 at 37° C.; 4 h later, hydroethidine (10 μM) was added to cells and incubated for 15 min at 37° C. Fluorescence was measured by flow cytometry. Dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) was used in D1 cells expressing induced Fhit, stressed with H_2O_2 (0.1 to 1.0 mM), treated with 10 μM DCFH-DA, and incubated for 1 h at 37° C. DCF fluorescence was measured by flow cytometry on a FAC-Scan flow cytometer and fluorescence microscopy.

Hsp60 and Hsp10 Silencing—A549 lung cancer cells at 8×10^5/well (6 wells plate) were transfected by Lipofectamine 2000 reagent (Invitrogen) and 6 μg of Hsp60 and/or Hsp10 siRNAs (Dharmacon catalog numbers NM_002156 [GenBank] and NM_002157 [GenBank], respectively); 48 h later cells were infected with AdFHIT at m.o.i. 1 and collected for cytosol/mitochondria protein fractionation 24 h later. Proteins were analyzed by SDS-PAGE and immunoblotting; filters were probed with Hsp60, Hsp10, and Fhit antisera. Protein loading was normalized with GAPDH and CoxIV. 1×10^6 H1299 D1 and E1 lung cancer cells were transfected as described above and at 24 h after transfection the cells were PonA-induced; 48 h after induction a cycloheximide (CHX) (10 μg/ml) chase at 1, 4, 6, and 12 h was performed and the protein lysates were analyzed as described herein.

Real-time RT-PCR—Total RNA isolated with TRIzol reagent (Invitrogen) was processed after DNase treatment (Ambion) directly to cDNA by reverse transcription using SuperScript First-Strand (Invitrogen). Target sequences were amplified by qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems). FDXR primers were: forward, 3'-TCGACCCAAGCGTGCCCTTTG-5' [SEQ ID No. 24]; reverse, 3'-GTGGCCCAGGAGGCGCAGCATC-5' [SEQ ID No. 25]. Samples were normalized using Actin and GADPH genes.

Chemotherapeutic Drug Treatment—Paclitaxel (Sigma) was dissolved in DMSO as a 10 mmol/liter stock solution and stored at −80° C. Cisplatin (Sigma) was dissolved in water and freshly prepared before use. H1299 D1 and E1 cells were seeded (1×10^4 cells/well) in 96-well culture plates, PonA-induced, and after 24 h treated with paclitaxel (50, 100, and 500 ng/ml) or cisplatin (0.05, 0.1, and 0.2 mM). H1299 D1 and E1 cells PonA-induced were incubated for 24, 48, and 72 h and assessed for viability with an MTS kit (Cell Titer 96® Aqueous MTS kit, Upstate Biotechnology, Lake Placid, N.Y.), as recommended by the manufacturer.

Example III

Generation of recombinant adenoviruses—The recombinant adenovirus carrying the wild-type FHIT cDNA (AdFHIT) was prepared as previously described (Ishii et al, 2001 Cancer Res 61:1578-1584). A His-tagged FHIT cDNA was generated by PCR with the following oligonucleotides: 5'-ACgTggATCCCTgTgAggACATgTCgTTCAgATTTggC-3' (forward) [SEQ ID NO: 26] and 5'-TTgTggATCCTTATCAgTgATggTgATggTgATgCgATCCTCTCTgAAAgTAgCCCgCAg-3' [SEQ ID NO: 27]. These primers were designed with a BamHI restriction site for subcloning into the transfer vector pAdenoVator-CMV5-IRES-GFP. The Ad-His6 was generated with the AdenoVator™ kit (Qbiogene, Carlsbad, Calif.), following the manufacturer's procedure. Ad GFP, used as control, was purchased from Qbiogene (Carlsbad, Calif.).

Generation of a recombinant expression vector carrying FDXR cDNA-Wild-type ferredoxin reductase full-length was amplified from human brain cDNAs (Clontech, Palo Alto, Calif.) with primers: 5'-CTgTTCCCAgCCATggCTTCgCgCTg-3' (forward) [SEQ ID NO: 28] and 5'-TCAgTggCCCAggAggCgCAgCATC-3' [SEQ ID NO: 29]. The amplification products were subcloned into the pcDNA3.1/V5-HisTOPO TA vector (Invitrogen, Carlsbad, Calif.). Sequencing excluded mutations in the amplified products.

For the preparation of a V5-tagged ferredoxin reductase cDNA, PCR amplification was performed with the same primer sequences with the exclusion of the FDXR physiological stop codon in the reverse primer. That is, both wild-type and V5-tagged ferredoxin reductase (FDXR) cDNAs were prepared by using as a template the wild-type coding sequence of the human ferredoxin reductase gene (GenBank Accession # NM_024417). The ferredoxin reductase coding sequence was amplified from human brain cDNAs (Clontech).

The primers used to generate the V5-tagged FDXR cDNA were: Forward: 5'-CTgTTCCCAgCCATggCTTCgCgCTg-3' [SEQ ID NO: 30]; and Reverse: 5'-gTggCCCAggAggCgCAgCATC-3' [SEQ ID NO: 31]. It is to be noted that the oligonucleotide sequences are identical except for the reverse primer used for the generation of the V5-tagged cDNA, where the physiological stop codon of the FDXR was omitted in order to fuse in frame the FDXR coding sequence with a V5-tag.

The amplification products were subcloned into the pcDNA3.1/V5-HisTOPO TA vector (Invitrogen, Carlsbad, Calif.). Sequencing excluded mutations in the amplified products.

In certain embodiments, the adenovirus being capable of isolating Fhit-His6, comprises an adenovirus carrying a FHIT-His6 cDNA. The FHIT-His6 cDNA can be prepared by using as a template the wild-type coding sequence of the human FHIT gene (GenBank Accession # NM_002012). In order to introduce a polyhistidine-tag at the C-terminus of final Fhit product, a PCR amplification of the wild-type FHIT coding sequence was carried out with a reverse primer designed to abolish the physiological stop codon and to add to the endogenous FHIT sequence a stretch of 18 by coding for six hystidines followed by an artificial stop codon. Furthermore, both forward and reverse primers carried a BamHI restriction site for an easy subcloning. The oligonucleotide sequences used for this amplification were the following: Forward: 5'-ACgTggATCCCTgTgAggACATgTCgTTCAgATTTggC-3' [SEQ ID NO: 26]; and Reverse: 5'-TTgTggATCCTTATCAgTgATggTgATggTgATgCgATCCTCTCTgAAAgTAgACCCgCAg-3' [SEQ ID NO: 27]. The PCR amplification product was sequenced to exclude random mutations before to be cloned in an Ad5 recombinant genome (AdenoVator™, a vector purchased by Qbiogene).

In certain embodiments, a method of isolating exogenously over-expressed Fhit-His6 includes using an adenovirus carrying a FHIT-His6 cDNA wherein the Fhit-His6 is isolated through the His tag. Fhit-His6 represents the recombinant protein whose expression is driven into a mammalian cell through the Ad FHIT-His6 vector. The His6 epitope allows for the recovery of the recombinant Fhit-His6 protein plus the protein complex interacting with the recombinant protein itself by taking advantage of the Ni-NTA system. This system is commercially available from Qiagen. Briefly, human A549 cancer cells were infected with Ad FHIT-His6; forty-eight hours after infection, photo-cross-linking of intracellular protein complexes was performed with the cross-linker dithiobis (succinimidyl propionate) [DSP] purchased from Pierce in order to stabilize protein complexes in living cells. Cells were disrupted in a protein extraction buffer and Fhit-His6 protein complex was isolated with the Ni-NTA magnetic-bead technology by taking advantage of the great affinity of the His6 tag for such beads. The isolated Fhit-His6 protein complex was then investigated through mass spectrometry to identify all proteins present in the complex.

In certain embodiments, a recombinant adenovirus carrying fragile histidine triad (Fhit) FHIT cDNA can be modified at its 3' with a sequence encoding a histidine-six epitope tag (AdFHIT-His6).

In certain embodiments, a method for mediating an apoptotic process in at least one cell, comprises exposing the cell to a fragile histidine triad (Fhit) gene product in an amount sufficient to mediate the apoptotic process in the cell.

In certain embodiments, a method for inducing an apoptosis process in a cell, comprises exposing the cell to a fragile histidine triad (Fhit) gene product in an amount sufficient to cause generation of reactive oxygen species (ROS) in the cell.

In certain embodiments, a method for mediating an apoptotic process in at least one cell, comprising: exposing the cell to a sufficient amount of fragile histidine triad (Fhit) gene product to allow the Fhit to enter mitochondria in the cell and to interact with Fdxr protein in the cell and to cause an increase in Fdxr protein level that is associated with generation of ROS, and causing a change in the apoptotic process in the cell.

It is to be noted that in previous studies, it was extensively proved that Fhit protein overexpression in Fhit-negative cancer cells is able to trigger programmed cell death (or apoptosis). In the instant invention, the inventors provide a rationale about the role of Fhit protein in the process of apoptosis. In fact, FHIT gene therapy of cancer cells performed with Ad FHIT (at the multiplicity of infection 50 or MOI50, i.e., 50 viral particles per cell) is responsible of Fhit overexpression; then, the newly synthesized recombinant protein is taken by its interactors Hsp60/Hsp10 from the cytosol to the mitochondria where Fhit interacts with FDXR (ferredoxin reductase) a protein belonging to the respiratory chain. This interaction leads to the mitochondrial generation of ROS (Reactive Oxygen Species). ROS represent the early step for the initiation of the intrinsic (or mitochondrial) pathway of the apoptotic process; in fact, they induce a damage in the mitochondrial membranes that, in turn, release cytochrome c into the cytosol. This step is crucial for the execution of apoptosis, as cytochrome c contributes with other cytosolic molecules (i.e., Apaf-1 and pro-caspase 3) to the generation of the apoptosome, a multiprotein complex able to drive the cell, in a non-reversible fashion, to apoptosis.

A method commonly used to study apoptosis consists in the detection of mature caspase-3 (an indicator of incipient apoptosis) by flow cytometric analysis (Becton Dickinson) [for reference, see Trapasso et al., 2003, PNAS, 100, 1592-1597].

In certain embodiments, a method for preparing a V5-tagged ferredoxin reductase cDNA, comprises PCR amplifying with primer sequences: 5'-CTgTTCCCAgCCATggCTTCgCgCTg-3' (forward) [SEQ ID NO: 28]. and 5'-TCAgTggCCCAggAggCgCAgCATC-3' [SEQ ID NO: 29] and subcloning the amplification products pcDNA3.1/V5-HisTOPO TA vector.

Both wild-type and V5-tagged ferredoxin reductase (FDXR) cDNAs were prepared by using as a template the wild-type coding sequence of the human ferredoxin reductase gene (GenBank Accession # NM_024417). The ferredoxin reductase coding sequence was amplified from human brain cDNAs (Clontech). That is, the primers used to amplify the wild-type FDXR cDNA were: Forward: 5'-CTgTTCCCAgCCATggCTTCgCgCTg-3' [SEQ ID NO: 28]; Reverse: 5'-TCAgTggCCCAggAggCgCAgCATC-3' [SEQ ID NO: 29].

The primers used to generate the VS-tagged FDXR cDNA were: Forward: 5'-CTgTTCCCAgCCATggCTTCgCgCTg-3' [SEQ ID NO: 30], and Reverse: 5'-gTggCCCAggAggCgCAgCATC-3' [SEQ ID NO: 31]. Note that the oligonucleotide sequences are identical except for the reverse primer used for the generation of the V5-tagged cDNA, where the physiological stop codon of the FDXR was omitted in order to fuse in frame the FDXR coding sequence with a V5-tag.

Finally, the two products were subcloned in the pcDNA3.1/V5-HisTOPO TA expression vector (purchased from Invitrogen) and then sequenced to assess that both products had no random mutations.

In certain embodiments, a method for generating a recombinant adenovirus, comprises preparing a recombinant adenovirus carrying the wild-type FHIT cDNA (AdFHIT); and generating a His-tagged FHIT cDNA using PCR with the following oligonucleotides: 5'-ACgTggATCCCTgTgAggACATgTCgTTCAgATTTggC-3' (forward) [SEQ ID NO: 26], and 5'-TTgTggATCCTTATCAgTgATggTgATggTgATgCgATCCTCTCTgAAAgTAgACCCgCAg-3' [SEQ ID NO: 27]. The FHIT-His6 cDNA was prepared as described herein. The recombinant adenoviral vector Ad FHIT-His6 was prepared according to the manufacturer's suggestions

(Qbiogene). Briefly, the amplified FHIT-His6 PCR fragment was digested with BamHI and subcloned into the BamHI linearized transfer vector pAdenoVator-CMV5-IRES-GFP. The pAdenoVator-CMV5-IRES-GFP/FHIT-His6 was co-transfected with the E1/E3 deleted Ad5 backbone viral DNA into 293 cells. Viral plaques were screened for the presence of the Fhit-His6 protein by Western blot with specific penta-His antibodies (Qiagen). One positive clone was plaque-purified and amplified on 293 cells. After freeze/thaw cycles, the adenoviruses in the supernatant were purified on two successive cesium chloride gradients. The recombinant adenovirus was titered by the TCID50 method and aliquoted. Virus stocks were stored at −80° C. Finally, the recombinant adenovirus carrying the wild-type FHIT cDNA (Ad FHIT) was previously generated by Trapasso et al. (2003, PNAS, 100, 1592-1597).

Mitochondrial localization studies—Confocal microscopy was used to assess Fhit protein distribution by immunofluorescence; H1299 D1 cells, with inducible FHIT cDNA, and E1 cells, with empty vector, were treated with PonA for 48 hr, and living cells were stained with Mitotracker Red 580 (M−22425, Molecular Probes, Eugene, Oreg.) at a working concentration of 500 nM for 40 min under growth conditions. The cells were fixed and permeablilized by incubation in ice-cold acetone for 5 min and then washed in PBS. Cells were incubated for 1 hr with 5% BSA to block non-specific interactions and than incubated overnight with Fhit antiserum (Zymed, S. San Francisco, Calif.) at a working concentration of 1.6 μg/ml, washed with PBS and incubated with Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes). The slides were mounted in mounting medium for fluorescence with DAPI (Vector, Burlingane, Calif.) and visualized. For immunoelectron microscopy localization of Fhit, A549 cells infected with AdHis6 or AdFHIT, MOI 5, were fixed in 4% paraformaldehyde in PBS pH7.2 for 30 min at 4° C., washed 3 times with PBS, and remaining free aldehyde groups were reduced using a 30 min incubation in 0.05% sodium borohydride in PBS. Following a PBS wash, samples were blocked with 50 mM glycine in PBS for 30 min, washed twice in PBS, and dehydrated with 25 and 50% ethanol for 15 min each, followed by 3 changes of 70% ethanol for 15 min each. The samples were then infiltrated with 70% ethanol+LR White resin, hard grade (Electron Microscopy Sciences, Hatfield, Pa.) at 2:1 for 1 hr, 70% ethanol+LR White at 1:2 for 1 hr, 100% LR White for 1 hr and 100% LRW overnight at 4° C. The following day the cells received 2 more changes of 100% LR White and were polymerized in gelatin capsules at 58° C. for 20-24 hr. 900 nm thin sections were cut using a Reichert UCT Ultramicrotome and a diamond knife and placed on nickel grids. The grids were floated section side down on drops of PBS for 5 min, 5% goat serum in PBS for 1 hr at room temperature (RT), and either Penta-His mouse monoclonal antibody at 20 mg/ml (Qiagen, Valencia, Calif.) diluted in PBS containing 0.1% BSA and 0.05% Tween −20 (BSA/Tw) or BSA/Tw alone overnight at 4° C. in a humidified chamber. The following day the grids were washed 6× for 5 min each using PBS and then incubated with h goat anti-mouse 10 nm colloidal gold conjugate (Ted Pella, Redding, Calif.) diluted 1:10 in BSA/Tw for 2 hrs at RT. The grids were washed 6× each for 5 min with PBS, rinsed with DI H2O and post-stained with 2.5% aqueous uranyl acetate for 3 min. Images were collected on a Tecnai 12 electron microscope equipped with a US1000 Gatan 2K digital camera.

Sample digestion and LC-MS/MS analysis—Proteins isolated with Ni-NTA beads were precipitated with cold acetone and resuspended in 6 M urea buffered at pH 8 with 0.1 M Tris-HCl. Protein reduction and alkylation was achieved, respectively, by the addition of DTT (final concentration 10 mM, 1 hr incubation at 37° C.) and iodoacetamide (final concentration 25 mM, 1 hr incubation at 37° C.). After neutralizing excess iodoacetamide with DTT (additional 5 mM), urea concentration was lowered to 1.5 M by dilution with 1 mM $CaCl_2$. Overnight digestion was carried out using 50 ng of TPCK-treated trypsin (Sigma). Total digestion solution volume was 100 μl.

Chromatography was performed on an Ultimate nano LC system from Dionex (Sunnyvale, Calif.). Digest mixtures (30 μl) were directly injected onto a Pepmap C_{18} RP cartridge (0.3 mm ID×5 mm length) and washed for 10 minutes with H2O/trifluoroacetic acid (TFA)/acetonitrile 97.9:0.1:2 (v/v/v) before the RP trap was switched on-line to a 75 μm×150 mm Pepmap C_{18} nano LC column. Gradient elution of peptides was achieved at 300 nl/min using a 45-min linear gradient going from 5% B to 50% B. Mobile phase A was H 20/acetonitrile/formic acid (FA)/TFA 97.9:2:0.08:0.02 (v/v/v/v); mobile phase B was H2O/acetonitrile/FA/TFA 4.9:95:0.08:0.02 (v/v/v/v).

MS detection was performed on an Applied Biosystems (Framingham, Mass.) QSTAR XL hybrid LC-MS/MS operating in positive ion mode, with nanoelectrospray potential at 1800 V, curtain gas at 15 units, CAD gas at 3 units. Information-dependent acquisition (IDA) was performed by selecting the two most abundant peaks for MS/MS analysis after a full TOF-MS scan from 400 to 1200 m/z lasting 2 seconds. Both MS/MS analyses were performed in enhanced mode (2 seconds/scan).

LC-MS/MS Data Analysis—MS/MS spectra were searched by interrogating the Swiss Prot database on the Mascot search engine (www.matrixscience.com) (accessed on June 2006). The following search parameters were used. MS tolerance: 50 ppm; MS/MS tolerance: 1 Da; methionine oxidized (variable modification); cysteine carbamidomethylated (fixed modification); enzyme: trypsin; max. missed cleavages: 1.

Protein lists obtained from, respectively, both A549 infected with Ad FHIT-His6 and control were compared, and proteins exclusively present in the A549-Ad FHIT-His6 list were kept for further validation. As a first validation procedure, LC-MS/MS raw data were inspected using selected ion chromatogram (SIC) displaying mode. By SIC comparison, it could be assessed the exclusive presence of the peptides of interest, identified as belonging to the six candidate proteins under examination, in the Ad FHIT-His6 sample. Such verification step already provided rather strong evidence for the specific capture of the six candidate protein. Also, these findings were further validated by biochemical and functional assays.

In accordance with the provisions of the patent statutes, the principle and mode of operation of this invention have been explained and illustrated in its preferred embodiment. However, it must be understood that this invention may be practiced otherwise than as specifically explained and illustrated without departing from its spirit or scope.

REFERENCES

The references discussed above and the following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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What is claimed is:

1. A method for inducing an apoptosis process in a cell, comprising

exposing the cell to an exogenous fragile histidine triad (Fhit) gene product in an amount sufficient to cause generation of reactive oxygen species (ROS) in the cell; and

administering an oxidative stress to the cell, wherein the oxidative stress comprises peroxide, thereby inducing an apoptotic process in the cell.

2. A method for mediating an apoptotic process in at least one cell, comprising:

exposing the cell to a sufficient amount of an exogenous fragile histidine triad (Fhit) gene product to allow the Fhit to enter mitochondria in the cell and to interact with Fdxr protein in the cell, whereby the Fdxr resists proteosomal degradation and accumulates in the cell, thereby causing an increase in Fdxr protein level that is associated with generation of ROS, and

applying an oxidative stress to the cell, wherein the oxidative stress comprises peroxide, causing a change in the apoptotic process in the cell.

3. The method of claim **1**, further comprising:

administering at least one chemotherapeutic agent.

4. The method of claim **3**, wherein the at least one chemotherapeutic agent is selected from the group consisting of: paclitaxel and cisplatin.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,911,998 B2
APPLICATION NO. : 12/739541
DATED : December 16, 2014
INVENTOR(S) : Carlo M. Croce and Francesco Trapasso

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

Column 1, Lines 13-15 replace the Government Support Clause with:
--This invention was made with government support under grant numbers CA 078890 and CA077738 awarded by the National Institutes of Health. The government has certain rights in the invention.--

Signed and Sealed this
Sixth Day of October, 2020

Andrei Iancu

Andrei Iancu
Director of the United States Patent and Trademark Office